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Interactions of Retroviral Capsid Proteins With Restriction Factors

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I, Mark Peter Dodding confirm that the work presented here is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

It is becoming increasingly clear that mammalian cells can express a variety of factors that limit retroviral replication. One class of these factors blocks replication at a stage post-entry but prior to nuclear import and integration of the viral genome. Members of this family are known as restriction factors. The best characterised member is the mouse gene *Fv1* which determines susceptibility to infection by MLV. *Fv1* has two alleles known as $Fv1^n$ and $Fv1^b$, whose restriction characteristics determine the host range of different MLV strains. It has become apparent that the phenomenon of restriction is not limited to MLV and murine cells. HIV-1, as well as MLV is restricted at a post-entry step in a number of primate species including rhesus macaques, African green monkeys and owl monkeys. The *Trim5* gene in these species is the main determinant of both HIV-1 and MLV restriction. Viral determinants of this restriction map to the virus capsid protein, however despite strong genetic evidence, no direct interaction has been shown between capsid and restriction factor using a variety of biochemical approaches.

This project began by aiming to understand why this might be the case. Experiments using a series of mutants defective for proper processing and assembly of capsid yielded evidence suggesting that the restriction factor binding site is in fact formed only when capsid is in its polymeric state in a mature virus, thus explaining why conventional approaches had failed to detect any interaction. This also provided insight into the mode of action of restriction factors, suggesting that they have evolved to target assembled mature virus entering the cytoplasm.

In order to better understand the context of the capsid/restriction factor interaction, cell biology studies using live-cell microscopy were initiated. These revealed that Trim5α is found in association with the microtubule network in highly motile cytoplasmic bodies, suggesting the possibility that the incoming virus might interaction with Trim5α in this context.

Furthermore, structural studies aimed at obtaining a better understanding of the structure of the capsid core have suggested new interactions which may be important for its formation, stability and consequent infectivity of the virus. These offer a potential new target for anti-retroviral therapies.

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CONTENTS

ABBREVIATIONS

AGM	African green monkey
AIDS	acquired immune deficiency syndrome
ALV	avian leukosis virus
APS	ammonium persulphate
BSA	bovine serum albumin
CA	capsid
CFP	cyan fluorescent protein
CTD	C-terminal domain
CypA	cyclophilin A
D-MEM	Dulbecco's modified eagles medium
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotidetriphosphates
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
eGFP	enhanced green fluorescent protein
EIAV	equine infectious anaemia virus
ELISA	enzyme linked immunosorbent assay
EM	electron microscopy
FV	Friend virus
GA	geldanamycin
Gag	group specific antigen
HIV-1	human immunodeficiency virus type 1
HTLV-1, -2	human T-lymphotropic virus
IN	integrase
IRES	internal ribosome entry site
MA	matrix
MHR	major homology region
MLV	murine leukaemia virus
MMTV	mouse mammary tumour virus
NC	nucleocapsid
NTD	N-terminal domain
OMK	owl monkey kidney
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PPT	polypurine tract
PR	protease
Q-PCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RRE	Rev respone element
RSV	Rous sarcoma virus
RT	reverse transcriptase
STE	sodium tris EDTA buffer
SU	surface glycoprotein
TBE	tris borate EDTA buffer
TEMED	N, N, N', N' – tetra-methyl-ethylenediamine
TGN	trans golgi network
TM	transmembrane
TRIM	tripartite mofif
TWEEN 20	polyoxyethylene sorbitan monolaurate
VSV	vesicular stomatitis virus
VSV-G	vesicular stomatitis virus glycoprotein envelope
YFP	yellow fluorescent protein

LIST OF FIGURES AND TABLES

SUPPLEMENTARY DATA

All supplementary data is supplied on the CD attached to the back cover of this manuscript. Either 'Apple Quicktime' on Macintosh or PC or 'Windows Media Player' on a PC platform can be used to view the data.

Apple Quicktime is available to download free of charge for both Mac and PC from http://www.apple.com/quicktime

Supplementary Videos 1 and 2

Motility of Trim5α Cytoplasmic Bodies

Human TE671 cells transduced with vectors carrying GFP-HuTrim5α were imaged at 1.325 second intervals for 60 frames as described in *Materials and Methods*. Supplementary video 1 shows untreated cells. Supplementary movie 2 shows cells treated with 66μM nocodazole.

Supplementary Videos 3 and 4

Association of Trim5α cytoplasmic bodies with the microtubule network

TE671 cells co-transduced with retroviral vectors carrying CFP-α-tubulin and GFP-Trim5α were imaged at 10 second intervals. CFP-α-tubulin is shown in red for ease of viewing.

CHAPTER 1

INTRODUCTION

Mammalian cells can express a variety of factors that limit retroviral replication. One class of these factors blocks replication at a stage post cell entry but prior to integration of the viral genome. The prototypic member of this family is the mouse gene *Fv1* which confers the host with resistance to infection by certain strains of Murine Leukemia Virus (MLV). More recently the *Trim5* gene has been shown to be capable of preventing infection by a variety of retroviruses dependent upon its species of origin. The study of these factors, as well as offering insight into the early events of the retroviral lifecycle may also offer new targets for anti-retroviral therapy.

This thesis will focus on characterising the interaction between these factors and their viral target - the capsid protein, with aim of understanding the consequences for the virus of such an interaction.

1.1 Retrovirus classification

Retroviruses are positive single stranded RNA viruses distinguished from other members of their family by their distinct mode of replication – their RNA is reverse transcribed to form double stranded DNA which is integrated into the host cell genome.

First discovered in the form of Avian leukosis virus (ALV) and Rous sarcoma virus (RSV) almost 100 years ago (Ellermann, 1908; Rous, 1911), the retroviral family has now grown large with examples found in most vertebrates. Particularly well characterised members of the family and principal subjects of this thesis are MLV and Human Immunodeficiency Virus Type 1 (HIV-1) – the causative agent of the current worldwide epidemic of Acquired Immunodeficiency Syndrome (AIDS). Retroviruses have been classified into seven genera – alpharetroviruses, betaretroviruses, gammaretroviruses, deltaretroviruses, epsilonretroviruses, lentiviruses and spumaviruses. Classification is dependent upon a number of factors including host range, sequence, genetic organisation, morphology, the presence or

absence of accessory genes and tRNA usage (Hunter et al., 2000). The presence of accessory genes above the universal complement of *gag, pol* and *env* defines a virus as 'complex'. A virus lacking accessory genes is described as 'simple'. With a few exceptions, the first 3 genera comprise simple retroviruses, the latter 4 are complex viruses

Under the classification shown, the two viruses of primcipal importance to this thesis (and therefore of primary focus throughout the remainder of this introduction), MLV and HIV-1, are described as a gammaretrovirus and lentivirus respectively. ALV and RSV are alpharetroviruses. Mouse mammary tumor virus (MMTV) is an example of a betaretrovirus. Human T-lymphtropic virus 1 and 2 (HTLV-1, -2) are deltaretroviruses. The fish virus, walleye dermal sarcoma virus is described as an epsilonretrovirus. As well as HIV-1, lentiviruses include simian immunodeficiency virus (SIV) and equine infectious anaemia virus (EIAV). Finally, simian foamy virus is the best characterised member of the spumavirus genus.

1.2 Retrovirus structure and life cycle

The following constitutes a general introduction to the retroviral lifecycle with specific emphasis on the phases most relevant to this thesis, with a focus on MLV and HIV-1, see figures 1.1 and 1.2.

1.2.1 Genomic organisation

Retroviruses possess two identical copies of a positive sense single-stranded RNA genome. This genome is capped at the 5' end with methylguanosine and is polyadenylated at the 3' end (Beemon and Keith, 1977; Furuichi et al., 1975)(Figure 1.2).

Coding regions

A simple retroviral genome encodes three genes, *gag, pol* and *env*. *Gag* encodes the internal viral structural components, *pol*, several enzymatic components and *env*, the viral envelope proteins (Figure 1.1). They are invariably arranged on the genome in this sequence in a 5' to 3' direction with varying degrees of overlap (Coffin and

Billeter, 1976; Coffin et al., 1997). In complex retroviruses, genes encoding accessory proteins are present, situated either between *pol* and *env*, or at the 3' end of *env* again often with overlap. In cell culture systems, genes encoding viral proteins can be supplied in trans.

This is particularly important for retroviral vectors where the viral RNA genome can then be used to encode a variety of other proteins.

Non-coding regions

The full RNA genome is flanked by regions known as R and U5 on the 5' end and R and U3 on the 3' end. These sequences are essential for the reverse transcription of viral RNA. The tRNA primer binding site (PBS) used for initiation of reverse transcription is found immediately after the U5 region (Verma et al., 1971). This is $tRNA_{pro}$ in the case of MLV or $tRNA_{lys}$ in the case of HIV-1.

During reverse transcription, these regions are used to generate the identical sequences known as long terminal repeats (LTR) on each end of the provirus composed of U3, R and U5 (Hughes et al., 1978). These LTR sequences serve as a promoter and regulatory region for the provirus – the transcription start site is found at the boundary of U3 and R in the 5' LTR with regulatory sequences within U3. The polyadenylation signal for both MLV and HIV-1 is found within the 3' R region, with the site of processing further downstream.

1.2.2 Viral proteins

The viral proteins are produced by translation of the RNA genome or one or more splice variants by the host RNA polymerase II.

The internal viral structural components are synthesised as the polyprotein Gag. Upon maturation, this is processed into its constituent parts by the viral protease. The viral enzymatic components are found in the *pol* region of the genome, and synthesised as Gag-Pol. Roughly 20 times more Gag is produced than Gag-pol (Jamjoom et al., 1976). For both MLV and HIV-1, Gag and Gag-Pol translation is initiated at the same start codon and most commonly terminates at the 3' end of Gag due to the presence of a stop codon. In only around 5 percent of translation events is Gag-Pol is produced. In the case of MLV, Pol is in the same reading frame as Gag. Read through the 3' Gag stop codon (MLV) allows translation of the full length Gag-

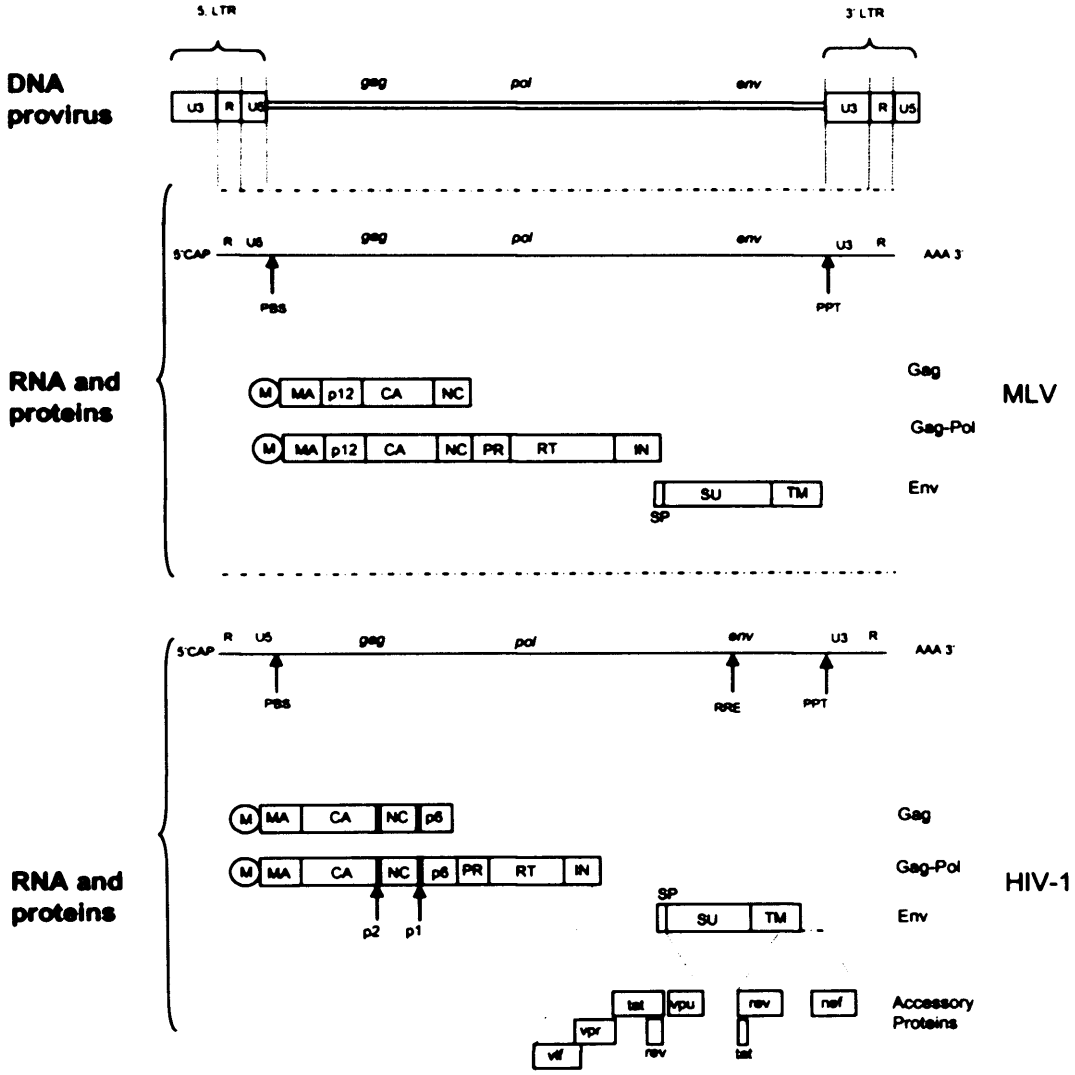


Figure 1.1 Schematic showing organisation of MLV and HIV-1 RNA genomes, DNA proviruses and viral proteins.

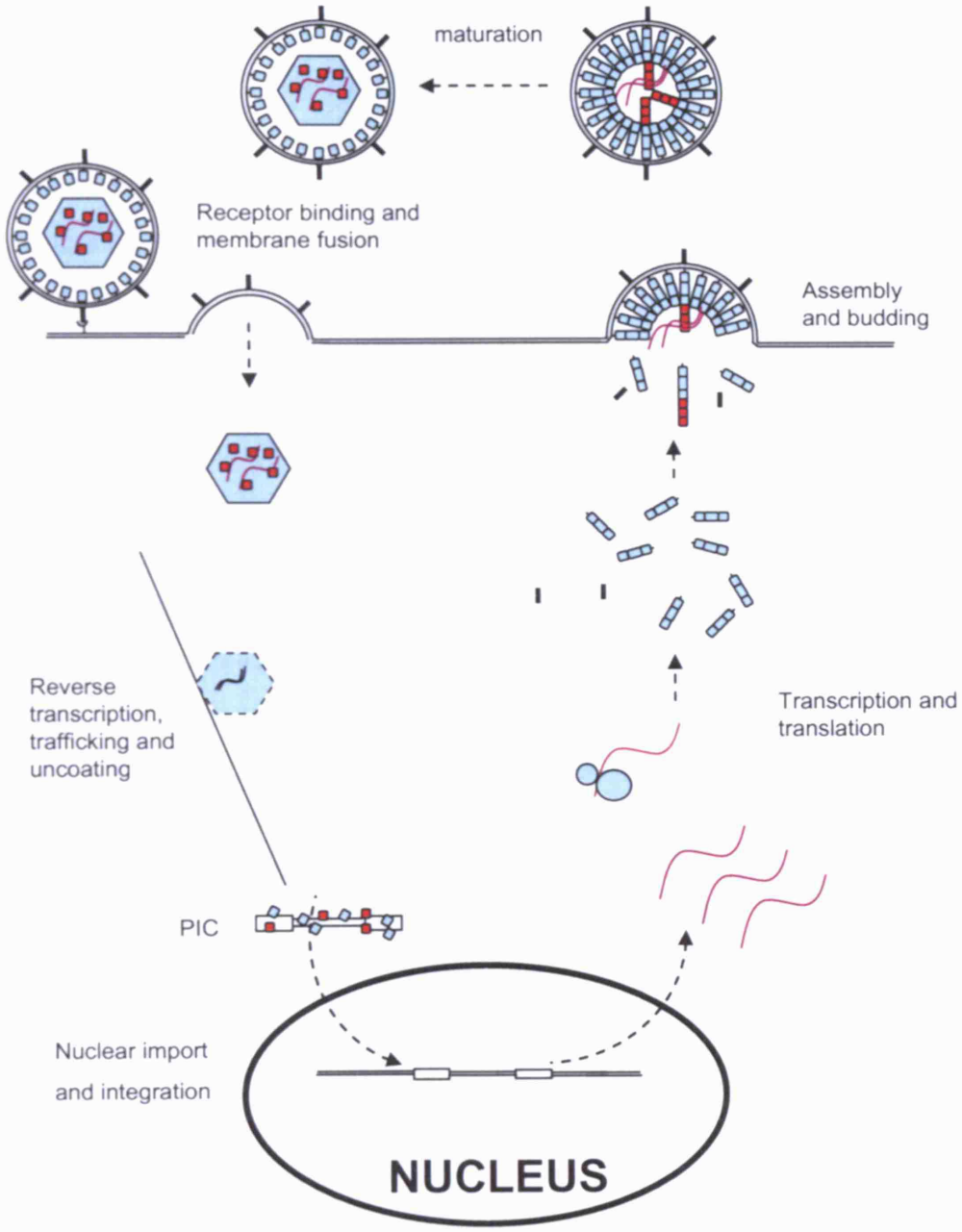


Figure 1.2 The retroviral lifecycle

Pol (Yoshinaka et al., 1985). In the case of HIV-1, Pol is not in frame with Gag but a -1 ribosomal frameshift (Jacks et al., 1988) allows the continuation of translation to produce Gag-Pol for a small proportion of translation events.

The organisation of the Gag polyprotein varies between genera, but all contain a matrix protein (MA), a capsid protein (CA) and nucleocapsid (NC). MLV contains an additional protein known as p12, found between MA and CA. HIV-1 encodes a protein known p6 found downstream of NC. In addition, short peptides are found at the carboxy and amino ends of HIV-1 NC known as p2 and p1 respectively.

The MA proteins of both HIV-1 and MLV are myristlyated on their amino termini. This post-translational modification, along with basic residues on its N terminus is involved in targeting Gag to membranes (Henderson et al., 1983; Rein et al., 1986; Saad et al., 2006; Veronese et al., 1988). MA may also be important for incorporation of envelope proteins into the virion via interactions with the cytoplasmic tail of TM. Some deletions in HIV MA can prevent this process (Yu et al., 1992). Contradicting this data, if the cytoplasmic tail of HIV TM (gp41) is shortened, Env is encorporated efficiently. In addition, it is possible to pseudotype viruses with hetrologous Env further indicating that this MA-Env interaction is not essential (Aiken, 1997; Soneoka et al., 1995).

NC proteins bind viral RNA via an interaction between Zn^{2+} finger motifs and the viral RNA genome and so are important for the packaging of viral RNA (Berkowitz et al., 1995). NC may also aid in RNA dimerisation and tRNA primer binding during the initiation of reverse transcription (Fu and Rein, 1993; Prats et al., 1988). Association between NC I domains is also essential for Gag assembly and is discussed in more detail later.

The most detailed studies of the p12 protein of MLV have been carried out by Goff and colleagues. Its disruption can affect both virion production and also early events in the virus lifecycle. The p12 protein contains a proline rich sequence (PPPY) known as a late (L) domain which is important for virus assembly. Disruption of other regions of this protein by point mutation can result in virions blocked either before or after reverse transcription indicating an important role for p12 in the early events of the lifecycle (Yuan et al., 2002; Yuan et al., 1999).

HIV-1 Gag has an additional domain known as p6. p6 is critical for virus release due the PTAP L domain found within it (Gottlinger et al., 1991).

The Gag-Pol polyprotein possesses three additional domains in the case of both MLV and HIV-1. Pol, like Gag, is processed by the viral protease into its constituent parts – protease (PR), reverse transcriptase (RT), and integrase (IN). RT is a RNA dependent DNA polymerase responsible for converting the RNA genome into the DNA form. IN mediates integration of the provirus into the host genome.

The viral envelope components (Env) are produced by translation of an mRNA from which the *gag-pol* region is removed by splicing. For both MLV and HIV-1, the splice donor site sits upstream of *gag*. The MLV splice acceptor site is found at the 3' end of *pol* within the region endoding integrase (IN). For HIV-1, the splice acceptor site which allows production of the *env* encoding RNA is found upstream of *vpu*. The resulting spliced RNA encodes both Env and Vpu. The other HIV-1 accessory proteins (discussed below) are produced by a multitude of splicing events with several other donor and acceptor sites. These events are described in detail by Rabson and Graves (Rabson and Graves, 1997).

Env is made up of two components, SU – the surface glycoprotein and TM - a transmembrane protein. Env translation begins on free ribosomes but a short hydrophobic signal peptide on the amino terminus of Env directs the nascent protein/ribosome to the rough endoplasmic reticulum (ER) where is it co-translationally inserted into the lumen of the ER. During this process the signal peptide is removed and the protein becomes heavily glycosylated on specific asparagine residues with the surrounding consensus sequence Asn-X-Ser and Asn-X-Thr (where X is any amino acid except proline) (Hunter and Swanstrom, 1990; Swanstrom and Wills, 1997). Hydrophobic amino acids with the TM domain of Env prevent the protein being entirely translocated into the lumen of the ER. The resultant polyprotein is cleaved by a cellular protease into its component parts in the Golgi apparatus and the Env complex is transported to the plasma membrane (Bedgood and Stallcup, 1992; Stein and Engleman, 1990; Wills et al., 1984).

The Env components are usually found in the form of a trimer on the surface of the virion (Einfeld and Hunter, 1988; Lu et al., 1995; Weiss et al., 1990). The SU protein is responsible for receptor binding upon contact with the host cell. The TM protein mediates membrane fusion between viral and host cell membranes allowing entry of the viral core into the cytoplasm. Receptor binding and membrane fusion mediated by Env are described below.

The capsid protein

Of particular importance to this thesis is the CA protein. CA is situated between p12 and NC in the case of MLV, or between MA and NC in the case of HIV-1. After processing and maturation, the CA protein condenses to form a distinctive electron dense core composed of a hexameric lattice forming a shell around the RNA and nucleocapsid components of the virion (Briggs et al., 2003; Li et al., 2000; Mortuza et al., 2004). This core is typically conical in the case of HIV-1 and roughly spherical in the case of MLV. The role of CA and the polymeric core it forms is not fully understood. Other enveloped RNA viruses such as vesicular stomatitis virus (VSV) (a rhabdovirus) or influenza (a myxovirus) do not possess such a shell.

Although its exact role, particularly in the early stages of the lifecycle in not fully understood, the importance of CA is clear. Disruption of several regions of CA can abolish infectivity and interfere with particle production. CA also contains a short sequence of amino acids known as major homology region (MHR) - the most highly conserved sequence in Gag. Disruption of this sequence by mutagenesis can affect the virus at several stages of its lifecycle, blocking either assembly or, if particles are produced, then early stages of the lifecycle (Craven et al., 1995; Mammano et al., 1994; Strambio-de-Castillia and Hunter, 1992).

Other than the MHR, amino acid conservation between CA proteins from retroviruses of different genera is poor. Despite this, secondary and tertiary structures appear very highly conserved supporting the notion of a critical role played by CA in the viral lifecycle. (Campos-Olivas et al., 2000; Cornilescu et al., 2001; Gamble et al., 1996; Mortuza et al., 2004).

CA mediates Gag-Gag contacts during virus assembly and appears to have an important role during the early stages of the viral lifecycle. Both are discussed in detail in the next section. The CA protein is also the target for the retroviral restriction factors Fv1 and Trim5α.

1.2.3 Assembly and maturation

Gag and Gag-Pol proteins are produced by translation of mRNA in the cytoplasm. In order to produce virus particles, Gag proteins must self associate and bud out of the cell. To produce an infectious virion, this particle must also incorporate viral RNA and possess the appropriate envelope proteins. The self-association of Gag molecules

is critical in this process. Gag expressed alone in cells is capable of self assembly and budding (Gheysen et al., 1989). After budding, the viral protease processes the Gag and Gag-Pol polyproteins into their constituent parts in a process known as maturation. This is essential for the production of an infectious particle. The regions of Gag required for these processes can vary dependent between genera (Swanstrom and Wills, 1997).

Domains of Gag involved in virus assembly

Several regions of Gag have been shown to have properties suggesting a role in self-association. Experiments designed to investigate these contacts have principally relied upon mutation or deletion of various parts of Gag followed by analysis of particle production.

The MA protein is capable of trimerisation, and indeed both HIV and SIV MA have been crystalised in that form (Hill et al., 1996; Rao et al., 1995). Despite this, it is clear that MA is not essential for particle production. Deletion of MA can still allow efficient release of assembled Gag and even replication competent virus particles providing a hetrologous myristylation targeting signal is supplied. (Accola et al., 2000; Lee and Linial, 1994; Reil et al., 1998). Taken together, these data suggest that the primcipal role of MA is in targeting Gag to membranes rather than any critical assembly function.

By contrast, CA seems to play a critical role in virus assembly. The CA protein is though to be composed of an N-terminal domain (NTD) and a C-terminal domain (CTD) separated by a flexible linker. For HIV-1 at least, it appears that the CTD makes the most important contacts in virus assembly (von Schwedler et al., 2003; Zhang et al., 1996).

The HIV CTD is capable of dimerisation in solution and has been crystalised in such a form (Gamble et al., 1997). Mutations designed to disrupt the dimerisation interface directly (W184A, M185A) or closely associated di-leucine motifs reduced Gag-Gag interactions *in vitro* and also reduced particle production (Burniston et al., 1999; Joshi et al., 2006; von Schwedler et al., 2003). Furthermore, disruption of a domain disordered in crystal structures, but predicted to be alpha helical, comprising the region at the C terminus of CA and the following SP1 region of HIV-1, can also interfere with particle production (Accola et al., 1998; Liang et al., 2003). A disruption of a predicted helix at the C-terminus of MLV CA can also disrupt

assembly (Cheslock et al., 2003). Finally, as described above, disruption of the MHR can cause a defect in assembly of HIV-1 and MLV. (Mammano et al., 1994; Provitera et al., 2001; Strambio-de-Castillia and Hunter, 1992).

The role of the NTD in virus assembly is somewhat less well defined. HIV-1 particle production (although entirely non-infectious), is still possible in the absence of the NTD (Accola et al., 2000). Despite this, mutations within the NTD can impact upon virus assembly. Disruption of residues between helices 4 and 6, specifically at positions 75, 76, 99, 100, 102, 107, 108, 110 and 112 by alanine substitution caused a reduction in HIV-1 particle production (Fitzon et al., 2000; von Schwedler et al., 2003).

In the case of MLV, disruption of the NTD can also affect virus assembly. Of particular relevance to this thesis, specific disruption of the NTD by insertion of 12 amino acids between positions 81 and 82 or 86 and 87 on a loop projecting laterally from the top of from helix 4 can block MLV assembly (Auerbach et al., 2006). This is discussed in great detail in chapter 6. Further, a mutation at position 119 reduced Gag-Gag interaction in yeast-2-hybrid experiments and also reduced particle production (Alin and Goff, 1996a; Alin and Goff, 1996b).

The involvement of NC in Gag multimerisation is likely driven by both protein-protein and protein-RNA interaction mediated by basic residues in the N terminus of NC described as the I (interaction) domain of HIV-1 (Swanstrom and Wills, 1997). Protein-RNA interaction may serve as a bridge between Gag molecules through the binding of several NC domains to the same RNA. These interactions may result in the concentration of Gag around RNA. Studies of HIV-1 show that disruption of NC basic residues can inhibit the ability of Gag to multimerise, produce particles and incorporate RNA. The protein-RNA interactions mediated by these basic residues are not necessarily specific for viral RNA. Disruption of the two Zn^{2+} finger motifs in HIV-1 NC which can specifically bind viral RNA has no effect upon assembly. (Cimarelli et al., 2000; Sandefur et al., 2000; Sandefur et al., 1998). In the case of HIV-1, the importance of NC in assembly is underlined by experiments showing that NC alone is as capable as full length Gag of interacting with full length Gag *in vitro*. This was reduced although not eliminated in the presence of RNase (Burniston et al., 1999). NC is also essential for MLV assembly. Deletion of NC blocks MLV Gag assembly. Mutation of N-terminal basic residues also inhibits virus production in a similar manner to HIV (Muriaux et al., 2004).

Organisation of Gag within the immature virion

Within the immature virion Gag is found in a closely packed hexameric array, with the Gag proteins arranged radially. The MA protein contacts the membrane whilst NC is found in the internal face of the sphere in association with the RNA (Briggs et al., 2006; Briggs et al., 2004; Huseby et al., 2005; Wilk et al., 2001).

Attempts to understand the arrangement of Gag in the immature structure have relied on three primcipal methods – assembly of His-tagged two dimensional structures on nickel chelating lipid membranes, analysis of particles produced in the presence of protease inhibitors, or *in vitro* assembly of recombinant Gag. In all three cases, assemblies are examined by electron microscopy and images reconstructed by averaging fourier transformations of the electron micrographs taking advantage of the repetitive structures within each image.

Using these methods, the centre-centre distance between hexamers has been estimated at around 80 angstroms using HIV-1 MA:CA:NC assembled on lipid membranes or in immature particles (Briggs et al., 2004; Huseby et al., 2005). This is a comparatively tighter packing in comparison to mature CA observed as helical tubes where the spacing appears greater, at 107 angstroms (Li et al., 2000), or a form of CA assembled in membranes suggested to correspond to the mature structure at, 90 angstroms (Mayo et al., 2003). A similar situation appears to be the case for MLV. A membrane assembled lattice suggested to correspond to the immature form was spaced at 63 angstroms whist the mature form was spaced at 91 angstroms (Mayo et al., 2003). Another mature form had inter-ring spacings of 80 angstroms (Ganser et al., 2003).

There does appear to be some variation in the measurements obtained for hexamer spacing depending upon the origin of the CA protein and also the method used to assemble the array. Despite this, it does appear clear that Gag is more tightly packed within the immature virion that CA is within the mature core.

Estimates for the number of immature Gag molecules within a virion vary depending upon the method used to make the estimate. However, the most recent data based upon cyro-EM analysis of immature virions places the figure at around 5000 (Briggs et al., 2004).

The molecular contacts important within the immature virion are likely related to those which drive self association and assembly although the precise details remain

to be defined. EM reconstructions have suggested that a dimeric CA CTDs and NTDs could make inter-hexamer contacts in the case of both HIV-1 and MLV (Huseby et al., 2005; Mayo et al., 2003). NC-NC interactions are probably also important although they have not been clearly observed by EM.

Taken together, these data are consistent with a model of multiple, synergistic, contacts throughout Gag mediating initial HIV-1 assembly. It may be that, NC-NC, NC-RNA, dimeric CA CTD interactions, as well as intra and inter-hexamer CA NTD contacts may combine to support a stable immature Gag lattice. Trimeric MA interactions may also support this structure but are not critical.

Budding

In most cell types, HIV and MLV bud at the plasma membrane, although in macrophages, the primcipal site of budding is thought to be multivesicular bodies which later fuse with the plasma membrane (Pelchen-Matthews et al., 2003). Membrane association is mediated by the N-terminal myristylation of MA and basic residues on its globular head (Ono and Freed, 1999; Ono et al., 2000; Rein et al., 1986; Schultz and Rein, 1989). Recent work has indicated that via its myristylate moiety, HIV-1 MA may specifically target particular lipid known as $PI(4,5)P_2$ (Ono et al., 2004; Saad et al., 2006). Binding of MA to this lipid triggers myristalate exposure. This also appears to be linked with MA trimerisation, with self association of Gag enhancing membrane association (Tang et al., 2004). Depletion of the plasma membrane of $PI(4,5)P_2$ resulted in retargeting of Gag to endosomal structures perhaps explaining the differences observed in location of budding in macrophages.

It has been suggested that HIV-1 may bud from cholesterol and sphingolipid enriched, detergent resistant, components of the plasma membrane known as lipid rafts (Ono and Freed, 2005). HIV-1 membranes are enriched in cholesterol as well a number of other putative raft components (Aloia et al., 1988; Aloia et al., 1993; Brugger et al., 2006; Nguyen and Hildreth, 2000) and appear to exclude the non-raft CD45 protein (Nguyen and Hildreth, 2000).

Both HIV and MLV Gag have also been found to interact with the kinesin family member Kif-4, implicating the microtubule network in Gag transport. The functional importance of this observation is not clear (Kim et al., 1998; Tang et al., 1999).

Retroviral budding is driven by the self association of Gag proteins targeted to membranes. The completion of budding from the plasma membrane – pinching off -

requires components of the host cell endocytic machinery. This interaction is mediated by so called late domains (L) within Gag (Freed, 2002). The HIV-1 late domain is located with in NC, consisting of a PTAP tetrapeptide. In MLV, this is found with p12 and consists of a PPPY tetrapeptide (Yuan et al., 2002; Yuan et al., 1999). In the case of HIV-1, this recruits the host protein TSG101 and a component of the endosomal sorting machinery, the ESCRT-1 complex (Garrus et al., 2001; Stuchell et al., 2004; VerPlank et al., 2001). Disruption of this domain or of the TSG101 results in a budding defect which manifests itself as particles tethered to the plasma membrane in the case of HIV-1. In the case of MLV, deletion of the PPPY motif caused the formation of chain structures composed of multiple virus particles on the cell surface (Yuan et al., 2000).

Processing and maturation

After budding from the plasma membrane, both MLV and HIV undergo a process known as maturation whereby the viral protease processes the components of Gag and Gag-Pol into its constituent parts and, of particular relevance to this thesis, releasing CA to form a condensed CA core. This process is required to produce a virion capable of infecting a new cell.

Retroviral proteases are aspartic proteases that function as dimers (Pearl and Taylor, 1987; Toh et al., 1985). How proteolysis of Gag is regulated so that it only occurs following assembly within the immature virion remains to be determined. It may be that only upon proper assembly with the immature virion are protease monomers properly orientated and in sufficient concentration to produce an active dimer.

Retroviral proteases target Gag at hydrophobic consensus sequences found at the junctions between the domains. In particular, the amino acids flanking the cleavage site, known as P1 and P1' are usually both hydrophobic. Mutation of P1 to a charged residue can in many cases abolish Gag processing (Oshima et al., 2004; Pettit et al., 2002).

For both MLV and HIV, processing appears to be an ordered, sequential process although with variations between each virus. In the case of MLV Gag, the first cleavage event appears to be between p12 and CA – at the amino terminus of CA (Naso et al., 1979; Yoshinaka and Luftig, 1977a; Yoshinaka and Luftig, 1977b). In the case of HIV Gag, the first event is at the N terminus of NC – between SP1 and

NC (Pettit et al., 1994). This is followed by processing between MA and CA, with the CA-SP1 event occurring later (Wiegers et al., 1998).

For both HIV-1 and MLV, processing at the N terminus of CA results in a conformational change. The N terminus of CA refolds to form a β-hairpin structure maintained by the presence of a salt bridge between the N terminal proline and an aspartate residue (Asp 51 in the case of HIV, Asp 54 for MLV). This refolding is essential for the formation of a condensed core (von Schwedler et al., 1998). The anti-retroviral drug PA-457, currently in clinical trials, inhibits maturation by preventing HIV-1 CA-SP1 processing (Li et al., 2003).

Exactly how a core forms upon maturation remains to be determined. One might propose two models. In the first, Gag processing would lead to an ordered condensation of CA from the immature form. In the second, the core would form spontaneously from the high concentration of free CA with the virion. The mature core contains only around a half to a third of the CA found with the mature virion (Briggs et al., 2004; Li et al., 2000). Using the first model, it would be difficult to explain how a majority of CA is excluded from the mature core. The second model therefore perhaps more likely, and is supported by the fact that HIV-1 conical cores can form spontaneously from recombinant bacterially produced CA:NC in the presence of nucleic acid (Li et al., 2000).

After processing by the viral protease and condensation of the CA core, a retrovirus is said to be in this mature form – a virion capable of infecting a new host cell (Figure 1.3) This mature form is a lipid enveloped particle of between 80nm and 100nm in diameter. The viral envelope proteins are found in association with the lipid membrane.

Of the proteins common to both HIV and MLV that previously formed domains of Gag, MA remains tethered to the lipid membrane whereas CA forms the condensed core around the RNA genome and nucleocapsid protein and enzymes necessary for a successful infection. This core is approximately spherical in the case of MLV or conical in the case of MLV.

Like the immature particle, the capsid core is believed to be composed of a hexameric lattice. The contacts that maintain this hexameric lattice are probably related to those which are involved in CA-CA interaction in assembly. Most models show the NTD forming the outer surface of the core, with the CTD less defined but appearing to be responsible for a dimeric inter-hexamer interaction. This is illustrated

Figure 1.3 A typical retrovirus

Illustration of a typical retrovirus particle. Enzymes and structural proteins are represented using X-ray crystal or NMR structure data from either MLV, HIV or SIV depending upon availability. (a) SU - SIV gp120 (b) TM - SIV gp41 ectodomain - associated with lipid bilayer (c) HIV matrix protein trimer (d) Capsid protein - NTD MLV hexamer, CTD HIV dimer (e) HIV nucleocapsid - associated with RNA genome (f) HIV protease (g) HIV reverse transcriptase (h) HIV integrase.
References (a) (Chen et al., 2005) (b) (Malashkevich et al., 1998) (c) (Malashkevich et al., 1998) (d) NTD (Mortuza et al., 2004) CTD (Gamble et al., 1997) (e) (De Guzman et al., 1998) (f) (Dreyer et al., 1992) (g) (Smerdon et al., 1994) (h) (Chen et al., 2000)

in figure 1.3. Evidence from assemblies of CA on membranes, analysis of *in-vitro* assemblies of CA, combined with crystal stucture data supports this model (Ganser et al., 2003; Li et al., 2000; Mayo et al., 2003; Mortuza et al., 2004).

In contrast to the immature particle, the absence of other Gag domains in the mature form, means that CA-CA interactions must be sufficient for maintaining the lattice. Perhaps this is at least part explained by the fact that is the proteolytic processing leads to refolding of the CA N-terminus, generating a β-hairpin structure. This hairpin forms critical interactions at the centre of the mature hexamer. (Mortuza et al., 2004; von Schwedler et al., 1998).

As described earlier, the process of maturation appears to alter the spacing between hexamers within the lattice. The centre-centre distances in the mature lattice appear to be greater. It may be that freed from constraints imposed by Gag, a conformational change occurs within CA. Data from Prevelige and colleagues suggest that upon maturation, a reorientation occurs between the NTD and CTD around the flexible region linking the two domains. The authors suggest that the CTD moves up to bind the NTD concurrent with a CTD translation in the X-Y plane of the lattice (Lanman et al., 2003; Lanman et al., 2004). Translation could explain the greater separation observed between hexamers.

1.2.4 Entry

HIV-1 and MLV enter their host cell by fusion between host cell membranes and the viral membrane. The exact mechanism of fusion for both HIV-1 and MLV remains to be completely determined but is believed to proceed in a similar manner to influenza HA mediated fusion (Skehel and Wiley, 2000). HIV-1 fusion is thought to primarily occur at the plasma membrane (Maddon et al., 1988; McClure et al., 1988; McClure et al., 1990; Stein et al., 1987) Attachment occurs through the trimeric SU Env protein to a host cell receptor. In the case of HIV-1, gp120 recognises CD4 on the surface of T-cells. This triggers a conformational change in gp120 which exposes the co-receptor binding site. Dependent upon the tropism of the virus, this is either CCR5 or CXCR4. Binding of the co-receptor triggers a subsequent conformational change in which TM (gp41) inserts into the target cell membrane causing the formation of a fusion pore. The trimer of gp41 forms a 6 helix bundle driving the fusion of viral and cellular membranes (Markosyan et al., 2003; Melikyan et al.,

2000). The formation of the fusion pore allows release of the viral core into the cytoplasm of the host cell. A new class of anti-retroviral fusion inhibitors can target this process. T-20 (enfuvirtide) is a peptide analogue of a region of gp-41, which targets formation of this helical bundle, preventing fusion.

MLV entry can occur either at the plasma membrane or via an endosomal pathway, dependent upon the nature of the Env gene. These have been distinguished by pH dependence or independence. Amphotropic MLV (a class of virus capable of infecting both rodent and non-rodent cell lines) appears to fuse at the cell surface (McClure et al., 1990) whereas ecotropic (a class of viruses only capable if infecting rodent cells) MLV appears to first enter the cell via endocytosis of its receptor (Kizhatil and Albritton, 1997). The MLV SU and TM domains are linked by a disulphide bond (Pinter et al., 1997). This is believed to maintain TM in a fusion inactive conformation. Binding of the receptor removes a stabilising Ca^{2+} inducing isomerisation of the disulphide bond, resulting in the loss of SU from the virus and triggering the fusion fuction of TM. In the case of ecotropic receptor, low pH may be required for this process. The ecotropic MLV receptor is mCAT-1, a cationic amino acid transporter (Albritton et al., 1989; Kim et al., 1991) The receptor for amphotropic MLV is known as Ram-1 (Miller et al., 1994). Both are transmembrane proteins with seven membrane spanning regions.

There has recently been some question over the true pH independence of some envelope proteins. The avian leukosis virus subgroup A (ALV-A) envelope was thought to be pH independent. It now however appears that receptor binding primes the envelope for a following pH dependent step (Mothes et al., 2000). Whether this is applicable to other envelopes is not completely clear, however if it were to be the case, then endocytosis and subsequent acidification of endosomes may be more broadly required then previously thought.

It should be noted that in this study, many viruses used are pseudotyped with the vesicular stomatitis virus glycoprotein envelope (VSV-G) (Soneoka et al., 1995). This is a pan-tropic envelope which allows entry of the virus core into in many cell types via an endocytic pathway (Aiken, 1997). Both MLV and HIV-1 can be pseudotyped with this envelope.

1.2.5 Reverse transcription, trafficking and the role of capsid uncoating

After entry of the virus core into the cytoplasm, a number of events must occur. These are commonly described as reverse transcription, CA uncoating and virus trafficking. The relationship of these events to each other and their timing is not at all clear. This phase of the lifecycle is particularly critical for this study as the virus CA is targeted by restriction factors during this time.

Mechanistically, reverse transcription is very well defined. However the role of CA uncoating in reverse transcription and viral trafficking is unknown. The process of reverse transcription is carried out by reverse transcriptase – an enzyme which possesses RNA dependent DNA polyermase activity, DNA dependent DNA polymerse activity as well as RNase H activity (Telenitsky and Goff, 1997). Reverse transcription begins by initiation from a specific tRNA bound to the PBS near the 5' end of the RNA genome and proceeds until the 5' end of the RNA is reached. The produces a short negative strand of DNA comprising mainly the R and U5 regions known as strong-stop DNA. In several experiments described in this thesis, this product is detected as an early product of reverse transcription. The RNA component of the RNA/DNA duplex produced is degraded by the RNase H activity of RT. This allows transfer of the minus strand RNA to the 3' end of the RNA mediated by the complementary R region. Synthesis of the minus strand then proceeds to the PBS at the 5' end of the RNA. The RNA component of the resultant RNA/DNA duplex is again digested away by the RNase H activity of RT, except in an RNase resistant region known as the poly-purine tract (PPT). This segment primes plus stand DNA synthesis which pauses after a portion of the tRNA primer is reverse transcribed producing a short positive DNA known as plus strand strong stop. RNase H removes the tRNA, exposing the PBS allowing it to pair with the PBS on the negative strand. Reverse transcription can then be completed with each strand serving as the template for the other resulting in a linear double stranded RNA with identical LTRs on the 5' and 3' ends.

After fusion, as well as completing reverse transcription, the incoming virus must move from the site of entry into the cytoplasm to the nucleus. This process may involve the host cell cytoskeleton. Fluorescently tagged intracellular HIV particles have been observed in association with the microtubule network and to move upon it from the cell periphery towards the microtubule organising centre (McDonald et al.,

2002). Despite this, treatment of cells with nocodazole does not reduce HIV-1 infectivity, perhaps suggesting that redundant transport mechanisms may exist (Bukrinskaya et al., 1998).

HIV-1 has also been shown to associate with the actin cytoskeleton via the MA protein. Treatment of cells with actin depolymerising drugs can reduce virus infectivity and prevent the completion of reverse transcription. This can be overcome however if the virus is pseudotyped with the VSV-G envelope which allows entry via an endocytic route. This has led to the suggestion that the MA-actin interaction may be involved in the penetration of the virus through cortical actin at the plasma membrane (Bukrinskaya et al., 1998). However, it may also be that actin is required for the proper localisation of receptor and co-receptor (Iyengar et al., 1998). This could also explain the effect of VSV-G pseudotpying.

The composition of the reverse transcription complex/pre-integration complex has been the subject of several studies. Early work by Varmus and colleagues showed that MLV DNA can be isolated in the form of a large nucleoprotein complex which possesses all of the requirements for integration activity *in vitro.* CA protein was also shown to be part of this complex (Bowerman et al., 1989). Later work by Fassti and Goff confirmed supports this data (Fassati and Goff, 1999). In the case of HIV-1, CA was not detected as a part of isolated complexes although it was possible to detect MA, RT, and IN (Bukrinsky et al., 1993; Fassati and Goff, 2001; Miller et al., 1997).

It has been suggested that after entry, the virus undergoes a process known as uncoating – the loss of the capsid protein, although the evidence for this is somewhat contradictory. Electron microscopy studies have failed to observe clearly conical intact cores with the cytoplasm of an infected cell (Grewe et al., 1990) although another study has shown complexes of at least the appropriate size if not shape, some of which apparently contain CA (McDonald et al., 2002). As described above, biochemical data is consistent with a rapid uncoating process, at least in the case of HIV-1. In both case, the sedimentation velocity of isolated complexes decreased with time, suggesting that an uncoating process does occur (Fassati and Goff, 1999; Fassati and Goff, 2001).

Experiments examining the stability of isolated HIV cores do strongly suggest that a regulated uncoating process must occur. Mutations that either enhance or decrease the stability of detergent stripped HIV cores *in vitro*, reduce viral infectivity and the production of DNA by reverse transcription (Forshey et al., 2002). This also

suggests that perhaps the processes of reverse transcription and uncoating may be coupled.

Recent data examining a virus with alanine substitutions at postions 63 and 67 in CA, showed that despite being competent for reverse transcription, this virus was incapable of nuclear import and integration suggesting perhaps a role for CA at a late stage of viral infection, or at the very least a role for CA in producing a particle capable of these processes (Dismuke and Aiken, 2006).

The HIV-1 accessory proteins Vif and Nef have also been implicated in uncoating. Vif has been shown to enhance the stability of HIV-1 cores (Ohagen and Gabuzda, 2000). Nef is a component of the viral core and appears to be required for an early step in the virus lifecycle. Virions lacking Nef produce less reverse transcription product and are less infectious, although this effect can be overcome by VSV-G pseudotyping (Aiken, 1997; Forshey and Aiken, 2003; Schwartz et al., 1995).

One might advance several explanations as to why a regulated uncoating process might be required. It may be that some disassembly is necessary for entry of dNTPs into the core thereby allowing reverse transcription, and in the case of HIV-1, to reduce the size of the complex to that which could pass through a nuclear pore. This may however need to be balanced by a requirement for a stable CA core for critical steps in reverse transcription such as initiation or strand transfer or intra-cellular transport.

1.2.6 Nuclear import and integration

After reverse transcription, the resulting DNA and associated proteins are known as the pre-integration complex (PIC). HIV does not require cell division for replication (Lewis and Emerman, 1994; Weinberg et al., 1991). The pre-integration complex is most likely actively imported into the nucleus via the nuclear pore complex. The exact mechanism of nuclear import is not clear although importin 7 has been implicated in this process (Fassati et al., 2003). Several HIV-1 PIC proteins have also been shown to possess nuclear localisation signals including MA, IN and Vpr (Bouyac-Bertoia et al., 2001; Fouchier et al., 1998; Heinzinger et al., 1994; von Schwedler et al., 1994). As well as this, a central PPT flap within the genome generated during reverse transcription has also been suggested as important (Zennou et al., 2000). In addition, MA has been shown to interact with the nuclear shuttling

protein VAN (Gupta et al., 2000). A recent attempt to remove all of the identified signals in HIV-1 by producing chimeras with MLV proteins still produced a virus capable of infecting non-dividing cells (Yamashita and Emerman, 2005). It may well be that these sequences are functionally redundant and that others may exist.

MLV cannot infect non-dividing cells. It has been suggested that MLV gains access to the nucleus and host cell DNA during cell division after dissolution of the nuclear membrane rather than during an active nuclear import process (Lewis and Emerman, 1994; Miller et al., 1990; Roe et al., 1993). Recent work however has suggested that CA may play a part in this difference between MLV and HIV-1. A chimeric virus where HIV-1 MA-CA was replaced by MLV MA-p12-CA was incapable of replication in non-dividing cells. This was not the case if only MA was replaced or p12 added alone (Yamashita and Emerman, 2004). The authors suggest that this difference may be due to the apparent faster uncoating of HIV-1 relative to MLV (Fassati and Goff, 1999; Fassati and Goff, 2001). Excessive amounts of CA associated with the genome may inhibit nuclear import.

Integration of the viral DNA into the host cell genome is catalysed by the viral integrase enzyme. These steps are now well characterised. First, both 3' ends of DNA are cleaved by 2 base pairs back to a conserved CA dinucleotide (this occurs within the cytoplasm of the host cell)(Fujiwara and Mizuuchi, 1988; Roth et al., 1989). The exposed 3' hydroxyl residues mediate a nucleophilic attack on a phosphodiester bond in the genomic DNA (Craigie et al., 1990). This results in nicks derived from 3 unpaired nucleotides and a 5' phosphate end on each end of the provirus These are degraded and repaired by the cellular DNA repair machinery (Majors and Varmus, 1981; Yoder and Bushman, 2000).

Although purified recombinant IN is sufficient to mediate both reactions *in vitro*, it is apparent that cellular factors are also involved (Van Maele et al., 2006). One of these factors is the host protein BAF (barrier to auto-integration factor) which was identified because it appeared to prevent autointegration of the MLV provirus *in vitro* (Lee and Craigie, 1994). It is also associated with the HIV-1 PIC (Chen and Engelman, 1998; Lin and Engelman, 2003). Recently, BAF has been shown to interact with the inner nuclear membrane protein emerin. This association is critical for the infectivity of HIV-1. The authors speculate that emerin may aid in engagement of chromatin by the PIC (Jacque and Stevenson, 2006).

1.2.7 Transcription and RNA Processing

Transcriptional of the proviral genome results in the production of a 5' capped and 3' polyadenylated RNA. This is driven by the 5' LTR which contains both promoter and enhancer elements. Transcription initiates at the boundary between U3 and R and is carried out by RNA polymerase II. Enhancer sequences vary between genera however all posess a TATA box upstream of the initiation site (Rabson and Graves, 1997). The 3' LTR is only capable of its full capacity for transcription initiation in the absence of the 5' LTR (Fung et al., 1981; Neel et al., 1981; Payne et al., 1981). The phenomenon is known as promoter occlusion. It is thought that the transcription complexes travelling from the 5' LTR may hinder initiation at the 3' LTR (Adhya and Gottesman, 1982; Cullen et al., 1984). Retroviral RNA is polyadenylated. The consensus sequence AAUAAA is found in the R region in the case of both MLV and HIV-1 although the site of polyadenlyation is further downstream.

To allow production of a number of viral proteins, both MLV and HIV-1 RNA is spliced by the host cell machinery. In the case of MLV and HIV-1, a splicing event produces the RNA which encodes the Env protein; The *Gag-Pol* region is removed by the joining of splice donor and acceptor sites upstream of *gag* and at the 3' end of IN. For HIV-1, a multitude of splicing events also allow the production of the HIV-1 accessory proteins (discussed below).

In order to produce Gag-Pol, as well as an RNA genomes for progeny virions, retroviruses must ensure that a proportion of their RNA remains unspliced. This is achieved through a combination of inefficient use of splice sites (Katz and Skalka, 1990), RNA elements which interact with host cell factors (Bray et al., 1994) and in the case of complex retroviruses, virally encoded proteins which assist in RNA export. In the case of HIV-1, Rev plays a critical role in this process and is discussed below.

1.2.8 HIV-1 accessory proteins

The genome of HIV-1 contains 6 accessory genes beyond the usual complement of *gag, pol* and *env*. These are *vif, vpr, tat, rev, vpu,* and *nef*. The functions of these genes are diverse and play a critical role in the virus life cycle. Many interact with host cell factors.

The Tat protein of HIV-1 is a potent enhancer of HIV-1 gene expression. It is believed to function primarily by increasing transcription. Tat binds a small RNA stem loop structural element known as TAR found at the 5' end of the genome (Dingwall et al., 1989; Hauber and Cullen, 1988). Tat recruits the host protein cyclin T. Cyclin T is a partner for the cyclin dependent kinase CDK9 (Wei et al., 1998). Recruitment of this kinase (part of the P-TEFb complex (Zhou et al., 1998)) causes phosphorylation of the CTD of the α subunit of RNA polymerase II, allowing the polymerase to move from an initiation to an elongation mode (Marshall et al., 1996; Marshall and Price, 1995).

The Rev protein also binds a RNA sequence of defined secondary structure known as the Rev Response Element (RRE). The role of Rev is to assist in the nuclear export of unspliced viral RNA (Felber et al., 1989; Hadzopoulou-Cladaras et al., 1989; Malim et al., 1989). The Rev protein has been found to shuttle between the nucleus and the cytoplasm in a transcription dependent manner (Meyer and Malim, 1994). This occurs via the nuclear pore complex and is dependent on importinβ, CRM1 and Ran-GDP/GTP. Rev associates with the RNA via the RRE along with CRM1 and Ran-GTP. This is transported to the cytoplasm , leading the the conversion of Ran-GTP to Ran-GDP and dissociation of the complex. Rev is then returned to the nucleus in a complex with importin β and Ran-GDP (Askjaer et al., 1998; Fornerod et al., 1997; Henderson and Percipalle, 1997; Izaurralde et al., 1997; Neville et al., 1997).

The Nef protein has a number of roles ascribed to it. It can down regulate CD4 expression on T-cells (Garcia and Miller, 1991). This may prevent Env interaction with CD4 on the cell surface or prevent infection by newly release particles (Benson et al., 1993) Nef maybe also involved in the early stages of the viral lifecycle as its presence can enhance viral DNA synthesis and has been shown to be a component of the viral core (Aiken and Trono, 1995; Forshey and Aiken, 2003). Nef can also down-regulate the surface expression of MHC-I molecule sugesting a role in immune evasion (Schwartz et al., 1996). A number of *nef* alleles from other primate lentiviruses can also down-regulate TCR-CD3, this has been linked to suppression of T-cell activation and non-pathogenic infection. It appears that HIV-1 Nef does not have this ability. This may begin to explain the high pathogenicity of HIV-1 in humans (Schindler et al., 2006).

The Vpu protein of HIV-1 appears to be involved in promoting the efficient release of virions (Geraghty and Panganiban, 1993; Yao et al., 1992). The requirement for Vpu is cell type dependent (Geraghty et al., 1994). Recent work has suggested that Vpu may overcome a cellular factor inhibiting efficient release of virions (Neil et al., 2006). Vpu is also involved in the dissociation of intra-cellular gp160-CD4 complexes and induces the degradation of CD4 (Willey et al., 1992a; Willey et al., 1992b).

The Vpr protein also appears to have a number of roles. Vpr expression causes cell cycle arrest at the G_2M phase (Jowett et al., 1995; Rogel et al., 1995). It has also been linked to nuclear import of the PIC via its nuclear localisation signal (Fouchier et al., 1998; Heinzinger et al., 1994). It has also been suggested to have pro-apoptotic activities. Vpr is packaged into virus particles and a fluorescently tagged version has been used to follow the incoming RTC with cell (McDonald et al., 2002).

Much progress has recently been made in understanding the role of the Vif protein, particularly in its ability to overcome the activity of APOBEC3G. Vif is only necessary for replication of HIV-1 in certain cell lines. Vif deficient virus produced in cells where Vif is required for infectivity is unable to infect either permissive (not requiring Vif) or non-permissive (requiring Vif) cell lines, implying that Vif is required for the production of infectious virus. This producer cell effect is dominant suggesting that the producer cells possess a factor which inhibits replication and that Vif can overcome this factor (Madani and Kabat, 1998; Simon et al., 1998). Screening of a cDNA library from non-permissive cells revealed such a factor, CEM15 (Sheehy et al., 2002). CEM15 was found to be a member of the APOBEC family of cytidene deaminases. CEM15 was later renamed APOBEC3G. APOBEC3G has been found to deaminate the minus strand of vif-minus HIV-1 DNA (Harris et al., 2003; Lecossier et al., 2003; Mangeat et al., 2003; Zhang et al., 2003). The deamination of cytidine results in the conversion to uracil in the minus strand. This leads to incorporation of adenine in the plus strand, therefore causing G to A hypermutation throughout the genome. APOBEC3G is incorporated into Vif-minus virions. Vif binds APOBEC3G and causes its proteasomal degradation (Conticello et al., 2003; Sheehy et al., 2003). It also appears that other members of the APOBEC family can inhibit HIV-1 replication. The activity APOBEC3F is dependent upon the absence of Vif, whereas the activity of APOBEC3B and the mouse APOBEC3 were not affected by Vif (Bishop et al., 2004a). It may also be the case that APOBEC

proteins can target viral RNA as well as DNA (Bishop et al., 2004b). Suprisingly, MLV appears to be resistant to activity from either mouse APOBEC3 or any of the human forms. (Bishop et al., 2004a). Despite the clear cytidine deaminase activity possessed by APOBEC proteins, recent work has suggested that this may not be absolutely necessary for antiviral activity. Study of chimeras between ABOBEC3F and APOBEC3G showed no correlation between this enzymatic activity and antiviral potency, suggesting that APOBEC proteins may function via an as yet undefined mechanism (Bishop et al., 2006a; Newman et al., 2005). Restriction of infection by APOBEC provides an example of an innate host cell defence against retroviral infection. Other innate host cell defences also exist. One particular class described as capsid dependent restriction factors are the main topic of this thesis.

1. 3 Retroviral restriction factors

It is becoming increasingly clear that mammals possess a variety of factors that act to limit retroviral replication. These were first noticed in mice, where it became apparent that certain strains showed varying susceptibility to infection by MLV. Genetic studies by Lilly and Pincus suggested several loci which could control infection by Friend MLV (Lilly, 1970; Lilly and Pincus, 1973). These appear to function by a variety of different methods, either affecting the virus or the host cell. These loci were named *Fv* - friend virus susceptibility factors. *Fv1* (the subject of this thesis) and *Fv2* are discussed in detail below. Another originally identified as *Fv3* appears to aid in mediating a strong immune response required for clearance of infection (Chesebro and Wehrly, 1979; Hasenkrug and Chesebro, 1997). The gene responsible has not been identified. The others function via non-immunological methods. *Fv4* prevents infection by ecotropic MLV (Odaka et al., 1981; Suzuki, 1975) by blockading the receptor. *Fv4* is itself a deleted endogenous ecotropic provirus (Ikeda et al., 1985; Ikeda and Odaka, 1984).

1.3.1 Fv1 and Fv2

During the 1960s it was noticed that inbred strains of mice showed varying susceptibility to induction of spleen focus formation by Friend virus (FV) – a

complex of the replication defective spleen focus forming virus (SFFV) and the replication competent helper virus Friend murine leukaemia virus (Fr-MLV) (Kabat, 1989; Odaka, 1969; Odaka and Yamamoto, 1965). Lilly was able to show that this effect was determined by two independently segregating genes designated *Fv1* and *Fv2*. Alleles for susceptibility and resistance were found at each locus. The *Fv1* gene for resistance was shown to be dominant in contrast to the *Fv2* gene where the susceptibility gene was dominant (Lilly, 1970). *Fv1* targets the Fr-MLV part of the FV complex (Lilly, 1970) whereas *Fv2* controls erythroblastosis induced by SFFV by binding of its gp55 protein to the *Fv2* gene product. This leads to constitutive activation of the erythropoietin receptor (EpoR) (Aizawa et al., 1990). *Fv2* has since been shown to encode a positive acting truncated form of Stk – Sf-Stk – a receptor tyrosine kinase required for Epor signalling which is lacking in resistant cells (Persons et al., 1999). It is not discussed further here.

Fv1 was shown to target the Fr-MLV part of the FV complex (Lilly, 1970). Simultaneously, *in-vitro* work by Hartley and Rowe showed that MLV strains could be divided into 3 groups dependent upon their ability to replicate in culture on cells derived from NIH or Balb/c mice. Some, described as N-tropic, were able to propagate on NIH cells but not Balb/c cells whereas others, described as B-tropic showed the opposite phenotype. Others, described as NB-tropic were able to replicate with equal efficiency on both cell lines (Hartley et al., 1970). Fr-MLV in the FV complex fell into the N-tropic class. Studies of cells derived from mouse crosses showed that this effect was dominant and controlled by a single genetic locus (Pincus et al., 1971). It was later shown that this *in-vitro* N-B effect was in fact controlled by the *Fv1* locus (Pincus et al., 1975).

Mice strains were shown possess several different alleles of Fv1. Of particular importance to this thesis, the allele found in NIH Swiss mice that allows replication of N-tropic virus is known as $Fv1^n$. That found in Balb/c mice which allows B-tropic virus replication is $Fv1^b$ (Lilly and Pincus, 1973). An allele with no activity was found in the Himalayan mouse *M.dunni* (Lander and Chattopadhyay, 1984).

1.3.2 The Fv1 gene and its product

The *Fv1* gene was cloned in 1996 and shown to be very distantly related to the Gag protein of an endogenous retrovirus of the MERV-L family. It was found on

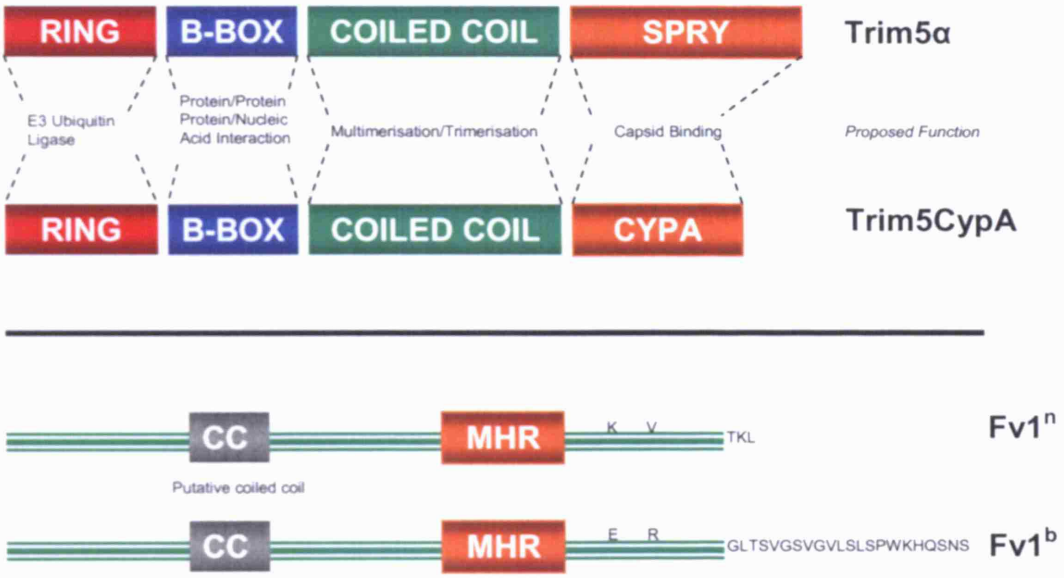


Figure 1.4 Schematic showing domain organisation of Trim5α, Trim5CypA and Fv1. The 4 domains of Trim5α/TrimCypA are shown, as well as their proposed activities.
$Fv1^n$ is shown contrasted to $Fv1^b$. Differences close to the C-terminus of the protein, the major homology region (MHR) and a putative coiled coil dimerisation domain are indicated.

chromosome 4 (Best et al., 1996; Stoye et al., 1995). In common with most retroviruses, it was found to possess an MHR (Figure 1.4)

$Fv1^n$ differed from $Fv1^b$ in several ways. Amino acids K and V at positions 358 and 399 in $Fv1^n$ were found to be replaced by E and R in $Fv1^b$. In addition, the C-terminus of $Fv1^b$ differed by 3 amino acids was well as being extended by another 19. $Fv1^n$ encodes a 440 amino acid protein whereas $Fv1^b$ has 459 amino acids. The *M.dunni* allele was shown to possess a premature stop codon, offering a plausible explanation of the the lack of activity (Qi et al., 1998).

An extensive study to map the domains of Fv1 responsible for restriction was undertaken by Bishop (Bishop et al., 2001). The extended C-terminus of $Fv1^b$ was dispensable for its activity and surprisingly, its deletion appeared to enhance restriction. Mutations made to disrupt the putative MHR completely abolish activity suggesting an important role for this domain in restriction. Progressive deletion of the N-terminus of the protein resulted in a gradual loss of activity cumulating in a total loss at position 92. Mutation of the variant residues at positions 358 and 399 and the C-terminal 3 amino acids altered specificity of restriction and activity although in a less than predictable fashion suggesting the specificity is determined in a complex, combinatorial fashion by these residues. Surprisingly, a large region within the middle of the protein was found to be dispensable for activity. Deletion of amino acids 123 to 227 still allowed complete restriction.

Various lines of evidence suggest that Fv1 contains sequences for multimerisation in both its N and C domains. Recent data has suggested that the N-terminus of Fv1 contains a putative coiled coil motif, disruption of which can also abolish restriction activity as well as interaction in mammalian-2-hybrid experiments (Bishop et al., 2006b)(Yap and Stoye, submitted).

Endogenous Fv1 is found associated with the trans golgi network (TGN) – a membranous organelle involved vesicle trafficking to and from the golgi (Yap and Stoye, 2003). When over expressed, puncta are observed on the TGN, suggesting that Fv1 may self associate at high concentrations. Mutations in the C terminal half of Fv1 appeared to disrupt these puncta, perhaps hinting at a multimerisation domain in the C terminal region in addition to the N terminal half.

The relevance of this association with the TGN is not clear. There are no known signal sequences in Fv1 which would target it there. Three possibilities present themselves. i. The TGN association Fv1 is required for restriction and is the site of

restriction. ii. Fv1 is trafficked through the TGN but has another as yet undetected site of action – perhaps diffuse in the cytoplasm. iii. The TGN form of Fv1 represents a dead end accumulation. The only evidence on any point is the fact that the Fv1 deletion mutant Int-1 which mis-localises to the ER is inactive and also exerts a dominant negative effect over endogenous Fv1 perhaps suggesting that Fv1 is prevented from reaching the TGN by multimerisation with the inactive ER sequestered form.

1.3.3 The viral target of Fv1

The virus CA protein has been strongly implicated as the target for Fv1 by several lines of genetic evidence. NB-tropic viruses derived from a B-tropic virus force-passaged on NIH cells showed altered electrophoretic mobility of CA protein as well as corresponding genomic changes (Hopkins et al., 1977; Rommelaere et al., 1979). Studies using recombinant virus combining different portions of N and B tropic virus suggested that amino acids 109 and 110 were sufficient to determine tropism (DesGroseillers and Jolicoeur, 1983; Ou et al., 1983). Of these positions, 110 is thought to be most important (Kozak and Chakraborti, 1996), with an Arg to Glu change at the position allowing a switch from N to B tropism. More recent work has suggested that despite the critical importance of positions 109 and 110, amino acids at positions 82, 92, 95, 105, 114 and 117 could also affect tropism further implicating CA as the target for restriction and suggesting a possible binding surface (Lassaux et al., 2005; Stevens et al., 2004).

1.3.4 Mechanism of action of Fv1

Although the effects of Fv1 upon the viral replication have become increasingly well defined, the mechanism of action of Fv1 remains to be determined.
Initial experiments indicated that the mechanism of action was not dependent upon entry of the virus or the envelope protein. Viruses containing the VSV genome pseudotyped with an N-MLV Env gave identical titres in plaque assays on restricting and non-restricting cells (Huang et al., 1973; Krontiris et al., 1973). Later, reciprocal studies showed that pseudotyping N and B tropic MLV cores with VSV-G envelope protein also does not affect restriction (Yap and Stoye, 2003).

Based upon hybridisation studies, it was found that restricted cells had fewer copies of the integrated viral genome than non-restricting cells suggesting that Fv1 acts before viral integration however levels of un-integrated viral DNA were virtually identical, implying that the process of reverse transcription was not affected (Jolicoeur and Baltimore, 1976; Sveda and Soeiro, 1976). This suggested that Fv1 inhibits replication at a stage after reverse transcription but prior to integration of the proviral DNA. Whether proper nuclear entry of the viral genome occurs is not completely clearly. Reports suggested that normal amounts of linear viral DNA was found in the nuclear fraction of restricting cells suggesting that the block may in fact be to integration rather than nuclear import (Jolicoeur and Baltimore, 1976). Arguing against this proposition, viral nucleoprotein complexes isolated from the same cells were fully competent for integration *in vitro* perhaps suggesting that in fact nuclear import may be blocked (Pryciak and Varmus, 1992). In addition, the production of 2 LTR circles, often considered a marker for nuclear entry was absent (Brown, 1997). An association of the linear viral DNA with the nuclear envelope might explain this discrepancy.

It should be noted that these experiments do not necessarily give information about the stage of the lifecycle at which Fv1 interacts with the incoming virus: only the phenotypic consequence to the virus can be measured. It is plausible that an early interaction could occur that is sufficient to disrupt infection but is not manifested until a later stage.

1.3.5 Saturability of Fv1 restriction

MLV restriction by Fv1 is saturable. Infection of cells at a high multiplicity of infection can overcome the effect of the restriction factor (Bassin et al., 1980; Duran-Troise et al., 1977). Further, pre-treatment of cells with genome deficient restricted virions produced by a packaging cell line can allow infection several hours later by a second restricted virus (Boone et al., 1990). This suggests that the restriction factor or possibly a co-factor is present in limiting amounts. Consistent with this proposition, natural levels of Fv1 expression appear very low (Yap and Stoye, 2003).

1.3.6 Murine retroviral restriction In non-murine mammalian species

Both Southern blot and genomic analysis show that non-murine cells do not possess an Fv1 gene (Best et al., 1996)(Ellis, PhD Thesis, UCL). Despite this, human cells, as well as cells from several primate species including rhesus macque have been shown to restrict N-tropic MLV but not B-tropic MLV (Towers et al., 2000) in a manner analogous to $Fv1^b$. This restriction is directed against the CA protein and again position 110 plays critical role. This restriction factor was named Ref1. Like Fv1, restriction by Ref1 is also saturable by pre-treatment with restricted virus (Towers et al., 2002). Unlike Fv1, Ref1 restriction acts at a stage prior to reverse transcription.

1.3.7 Restriction of non-murine retroviruses

Prior to the characterisation of Ref1, post-entry blocks to lentiviral infection had been observed in rhesus macaque cells. These cells showed differential sensitivity to infection by HIV-1 and SIV, with HIV-1 appearing to be restricted. This was shown not to be dependent upon the entry mediated by the envelope as chimeric viruses with HIV structural proteins and SIV envelope were still blocked. In addition, viruses pseduotyped with the pan-tropic VSV-G envelope protein have a similar phenotype (Himathongkham and Luciw, 1996; Hofmann et al., 1999; Shibata et al., 1995). These barriers to infection appear generally to prevent cross species infection. HIV-1 titres are significantly reduced on cells of old world monkeys when compared to human cell lines whereas SIVmac titres are reduced on new world monkey cell lines (Hofmann et al., 1999).

Work by several groups went on to show that this restriction of retroviral infection shares a number of features in common with Fv1 and Ref1 restriction. This restriction appeared to be saturable and dominant. The restriction activity could be overcome at high multiplicity of infection and was present in heterokaryons of restricting and non restricting origin. Like Ref1 but in contrast to Fv1, this restriction acted before reverse transcription and was named Lv1 (Besnier et al., 2002; Cowan et al., 2002; Munk et al., 2002). Once again, restriction was shown to be dependent upon the virus CA protein, using chimeric HIV/SIV viruses in combination with site

directed mutagenesis. (Cowan et al., 2002; Hatziioannou et al., 2003; Owens et al., 2004; Owens et al., 2003).

1.3.8 Cross saturation of Ref1 and Lv1 restriction

Restriction of multiple, divergent retroviruses by both Ref1 and Lv1 was shown most clearly by Hatziioannou and colleagues. (Hatziioannou et al., 2003) Human cells restrict not only N-MLV but also the horse lentivirus, equine infectious anemia virus (EIAV). African green monkey (AGM) cells were shown to restrict N-MLV, EIAV, HIV-1, HIV-2 and SIVmac. As both Ref1 and Lv1 had been shown to be saturable, cross-saturation experiments were performed to determine whether these divergent activities were mediated by the same gene. Amongst several examples, HIV was shown to be capable of abrogating restriction of N-MLV in AGM cells and EIAV was capable of abrogating N-MLV restriction in human cells, with the converse true in each case. Importantly, non-restricted virus was incapable of abrogating restriction, nor was its infectivity enhanced by abrogation.

In general a restricted virus was capable of abrogating restriction of other restricted viruses in a particular AGM or human cell line suggesting strongly that the broad restriction activities mediated by either Ref1 or Lv1 are in fact mediated by the same gene (Hatziioannou et al., 2003).

The experiment showing that HIV-1 could abrogate N-MLV restriction in AGM cells suggested that perhaps that Ref1 and Lv1 could be alleleic variants of the same gene (Stoye, 2002).

1.3.9 Identification of *Trim5α* as the gene responsible for both Lv1 and Ref1 activity

In a landmark paper in 2004, the Sodroski group succeeded in identifying a gene by a screen of a cDNA library from rhesus macaque cells that when expressed in human cells, conferred resistance to infection by HIV-1. The cDNA recovered was found to encode the gene Trim5α – a member of the tripartite motif (TRIM) family of proteins. Trim5α found to localise to cytoplasmic bodies when overexpressed. Further work by a number of labs followed rapidly showing that both the Lv1 and Ref1 activities of human and primate cells are mediated by Trim5α (Hatziioannou et

al., 2004b; Keckesova et al., 2004; Perron et al., 2004; Yap et al., 2004). siRNAs directed against Trim5α could abolish or reduce both Ref1 and Lv1 activity, raising viral titres up to levels approaching those in non-restricting cells. Importantly, when Trim5α was added to non-restricting cells, the human form of the gene could restrict N-tropic MLV but not B-tropic MLV or HIV-1. The rhesus macaque and AGM alleles of Trim5α restricted both MLV and HIV-1 but not B-tropic MLV. In the case of MLV, a single amino acid change at position 110 in CA could determine susceptibility to infection (Perron et al., 2004).

1.3.10 Trim5α the TRIM family of proteins

Trim5α is a member of the TRIM family of proteins. They are also known as the RBCC family because of the domains common to all members; a ***r***ing domain, one or two ***B***-box domains and a ***c***oiled-***c***oil domain. Many also have an additional C-terminal domain known as a B30.2 domain (or SPRY domain) (Nisole et al., 2005; Reymond et al., 2001).(Figure 1.4, pg 27)

A large number of TRIM proteins were characterised in a comprehensive paper by Reymond. Almost all, including Trim5α, are capable of homomultimerisation via their coiled coil domains (as tested in a two-hybrid system) (Reymond et al., 2001). Alternative splicing is also common. Trim5 has at least 6 alternatively spliced isoforms of which Trim5α is the longest and the only one shown to have functional activity against retroviruses although the γ isoform has been shown to have a dominant negative effect on retrovirus restriction (Stremlau et al., 2004).

The functions of other members of the family although poorly characterised appear to be diverse and are involved in several human diseases. Trim18, also known as MID1, appears to be involved in midline development in mice. It is also mutated in Optiz G/BBB syndrome in humans – again resulting in midline defects (Quaderi et al., 1997). Pyrin (TRIM20) is mutated in familial Mediterranean fever (TheFrenchFMFConsortium, 1997; TheInternationalFMFConsortium, 1997). The Trim19 gene – also know as PML is implicated in promyelocytic leukaemia, where a chromosomal translocation resulting in its fusion with retinoic acid receptor alpha (RARα) leads to the acquisition of oncogenic activity (de The et al., 1991; Goddard et al., 1991; Kakizuka et al., 1991). PML has also been strongly implicated in antiviral activity. Over-expression of PML confers resistance to VSV and influenza

A virus (Chelbi-Alix et al., 1998). More controversial data suggested a role for PML in inhibiting HIV nuclear entry (Turelli et al., 2001) although work from two other groups failed to reproduce these data (Bell et al., 2001; Berthoux et al., 2003).
Finally, expression of Trim1 from AGM in human cells was shown to restrict N-tropic MLV but not B-tropic MLV, although to a lesser extent than Trim5α, showing that other members of the TRIM family of proteins could have a role in CA dependent restriction (Yap et al., 2004). Trim34 has also recently been shown to have a very limited effect upon HIV-1 (Zhang et al., 2006a).

1.3.11 Domains of Trim5α involved in restriction

Following the identification of Trim5α an enormous effort was made by several labs resulting in a bevy of publications suggesting that the C terminal B30.2 domain contains the primary specificity determinants for restriction (Liu et al., 2005; Nakayama et al., 2005; Perez-Caballero et al., 2005a; Sawyer et al., 2005; Song et al., 2005b; Song et al., 2005c; Stremlau et al., 2005; Yap et al., 2005). In particular, a single amino acid change in human Trim5α (R332P) can confer the ability to restrict HIV-1 (Yap et al., 2005).
The role of other domains of Trim5α is less clear. The Zn^{2+} coordinating RING domain of Trim5α has been shown to possess E3 ubiquitin ligase activity and is capable of self ubiquitination (Diaz-Griffero et al., 2006; Xu et al., 2003). Trim5α disrupted by mutations of Cys residues which prevent Zn^{2+} binding and abolish ubiquitin ligase activity of the RING domain are still capable of restricting HIV-1, albeit at a considerably reduced level. Mutation of the B-box domain by disruption of residues which coordinate Zn^{2+} eliminated antiretroviral activity and could exert a dominant negative effect over the endogenous protein (Javanbakht et al., 2005).
The coiled coil domain is involved multimerisation (probably trimerisation) of Trim5α. Deletion of the coiled coil eliminates restriction activity as well as any dominant negative effects (Mische et al., 2005; Perez-Caballero et al., 2005a; Reymond et al., 2001).

1.3.12 Cyclophilin in the HIV lifecycle and owl monkey Trim5

Cyclophilin and restriction

HIV-1 Gag was shown to bind to cyclophilin A (CypA) in yeast-2-hybrid screens by Luban and colleagues (Luban et al., 1993). Further work showed that CypA was specifically incorporated into virus particles and that inhibition of CypA using the cyclosporin A - a competitive inhibitor of CypA which occupies the substrate binding site – reduced HIV-1 replication on cells in culture (Franke et al., 1994; Thali et al., 1994; Walsh et al., 1992). CypA binds an extended loop found after helix 4 of the N-terminal domain of HIV CA (now known as the cyclophilin binding loop) and has been crystalised in such a form (Colgan et al., 1996; Gamble et al., 1996; Yoo et al., 1997).

CypA is a chaperone protein which catalyses the cis/trans isomerisation of peptidyl-prolyl bonds. However its normal physiological role is unknown. Upon HIV binding, residues 89 and 90 of CA occupy the active site of CypA. CypA is capable of catalysing the isomerisation of a Gly-89-Pro-90 bond in HIV (Bosco et al., 2002) although the relevance of this observation is not clear. It has so far been impossible to differentiate between the binding of CypA and its catalytic activity in its role in HIV infection, as any mutations in CypA which reduce enzymatic activity also reduce substrate affinity (Eisenmesser et al., 2005).

Despite the fact that CypA is incorporated into virus particles via association with Gag, it appears that the target cell CypA is responsible for effects on infectivity of the virus (Hatziioannou et al., 2005; Sokolskaja et al., 2004), with CypA required prior to reverse transcription (Braaten et al., 1996).

Determining the role of CypA in the virus lifecycle is complicated by the fact that the CypA binding site on CA appears to overlap with the region of CA responsible for interaction with restriction factors (Owens et al., 2004). Indeed, CypA may well play a role in modulating the sensitivity of incoming HIV-1 to restriction factors. CypA binding by HIV-1 in human cells may protect the incoming virus from recognition by Trim5α. If the CypA-CA interaction is disrupted by the competitive inhibitor cyclosporin A, HIV is capable of saturating Refl restriction in human cells, allowing infection by N-tropic MLV. In addition, a non-CypA binding mutant of HIV – G89V also shows an enhanced ability to saturate Refl restriction (Towers et al., 2003).

In contrast, the addition of cyclosporin A appears to have the opposite effect upon some monkey cell lines. In AGM cells and rhesus macaque cells, CypA appears to be required for efficient restriction by Trim5α (Keckesova et al., 2006).

Trim5CypA

The effects of disrupting CypA-CA interaction in monkey cells described here so far are relatively mild, generally with resulting infectivity between 50% and 10% of wildtype. A much more dramatic effect has been observed on cells derived from the Owl Monkey. In these cells, disruption of the CypA-CA interaction either by using cyclosporin A or mutation of CA results in an increased infectivity greater than 100 fold. The reason for this dramatic effect became apparent after the cloning of the Owl Monkey Trim5 gene (Nisole et al., 2004; Sayah et al., 2004). In this case, a CypA insertion is found upstream of the B30.2 domain of owl monkey Trim5, resulting in transcription and subsequent translation of a Trim5CypA fusion protein consisting of the RBCC domain fused to CypA. As described above, the B30.2 domain is thought to mediate binding and specificity if restriction. It appears that in this case, that function has been usurped by CypA. *In-vitro* experiments have shown that the Trim5CypA fusion protein binds to HIV CA in a CypA dependent manner (Nisole et al., 2004).

1.4 Placing this work in context

As will be clear from the preceding pages, the field of retroviral restriction has advanced rapidly over recent years. This project began close to the time that Trim5α was identified by Sodroski and colleagues. During the subsequent three years, huge advances have been made in understanding restriction, particularly by Trim5α. Many of these advances have impacted upon the work presented here. In order to place this work in its proper context alongside other work in the field, some recent work characterising cellular localisation of Trim5α, binding to CA and potential mechanisms of action is not described in this introduction. Where appropriate, these discoveries are described in the *Results* chapters or in the *Discussion* found at the end of this thesis.

1.5 Project aims

The overall aim of this project was to enhance our understanding of the interaction between retrovirus capsid proteins and host restriction factors. This could shed light on the mechanism of action of these factors.

Previous attempts by a number of groups to show any direct interaction between Fv1 and MLV CA using standard techniques such as yeast/mammalian-2-hybrid, GST-pull down or co-immunoprecipitation had failed (J.Stoye, personal communiation). This project began by attempting to address these issues by explaining the cause using the phenomenon of abrogation (the only clear way of showing interaction between virus and Fv1). Results described in chapters 3 and 4 suggested that the lack of interaction observed may be due to the fact that the restriction factor binding can only occur when CA is in its polymeric form within a retroviral core. Chapter 5 describes experiments using polymeric CA material designed to test this hypothesis and probe the mechanism of action of both Fv1 and Trim5α.

In parallel to these studies, data became available showing potential critical contacts involved in the formation and maintenance of the viral CA core. A study described in Chapter 6 was undertaken to examine the relevance of these contacts in CA-CA interaction and the viral lifecycle. This study suggested that contacts by the N terminal domain of CA may be critical in both assembly of the virus and proper formation of the CA core.

Finally, an important step understanding the mechanism of action of any host restriction factor is to understand their cell biology. Trim5α has previously been described to form puncta with the cytoplasm known as cytoplasmic bodies. Chapter 7 describes experiments designed to characterise these structures and their relevance to retroviral restriction.

CHAPTER 2

MATERIALS AND METHODS

2.1 Recombinant DNA

2.1.1 Polymerase Chain Reaction (PCR)

Polymerase chain reactions were carried out to amplify sections of DNA from plasmids. *Pfu* Ultra polymerase (Stratagene) was used in all cases. Primers were purchased from either Genosys or Oswell. A typical reaction consisted of 500ng template DNA, 1µl of each primer at 25µM, 1µl 10mM dNTPs, 5µl polymerase buffer, and 1µl polymerase made up to 50µl with H_20. Using a MJ Research, PTC 100 thermal cycler, reactions were subjected to a 2 minute denaturing step at 95°C, followed by 25 cycles of 95°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute per kb of sequence to be amplified. Reactions were completed by a 1 minute incubation at 72°C. Samples were subsequently analysed by agarose gel electrophoresis.

2.1.2 Site-directed mutagenesis of Gag-Pol expression vectors

Site directed mutagenesis was carried out according to the guidelines in the Quikchange mutagenesis kit (Stratagene). Briefly; complementary oligonucleotides typically 20-40 nucleotides in length were designed to target the region of interest whilst incorportating one or more nucleotide changes. A list of all primers used is shown in table 2.1. A typical reaction consisted of 10ng template plasmid DNA, 125ng of each primer, 50µM dNTP, 1µl (2.5U) *Pfu* Turbo Polymerase, and 5µl 10X polymerase buffer made up to 50µl with H_20. After a 30 second 95°C denaturing step, reactions were subjected to thermal cycling of 12-18 cycles of 30 seconds at 95°C, 1 min at 55°C and 1 minute per kb of template plasmid at 68°C. After thermal cycling, reactions were incubated for 1 hour at 37°C in the presence of DpnI to digest

Lab name	Mutation /Purpose	Sequences
MRB 38/39	MLV MAxp12	5'-CCCCGATCTGCCCTTGATCCTGCTCTTACCCC-3' 5'-GGGGTAAGAGCAGGATCAAGGGCAGATCGGGG-3'
MRB 40/41	MLV p12xCA	5'-CCACCTCTCGGGCTGACCCACTCCGTTTGGGG-3' 5'-CCCCAAACGGAGTGGGTCAGCCCGAGAGGTGG-3'
MPD 001/002	MLV CAxNC	5'-GGAATGAGCAAACTTAGGGCCACCGTAGTTAG-3' 5'-CTAACTACGGTGGCCCTAAGTTTGCTCATTTC-3'
TS 83/84	MLV RT active site D224E	5'-CCTGCTACAGTACGTGGAGGACATACTACTGGCC-3' 5'-GGCCAGTAGTATGTCCTCCACGTACTGTAGCAGG-3'
TS 81/82	MLV Protease active site D32L	5'-CCGTCACCTTCCTGGTGCTTACTGGGGCCCAACACTCC-3' 5'-GGAGTGTTGGGCCCCAGTAAGCACCAGGAAGGTGACGGG-3'
MPD AT/AU	MLV CA D54A	5'-CAGCCCACCTGGGATGCCTGCCAGCAATTATTAG-3' 5'-CTAATAATTGCTGGCAGGCATCCCAGGTGGGCTG-3'

MPD CD/CE	HIV CA E79R	5'-CAA TGA GGA AGC TGC AAG ATG GGA TAG ATT GCA TC-3' 5'-GAT GCA ATC TAT CCC ATC TTG CAG CTT CCT CAT TG-3'
MPD CB/CC	HIV CA R82D	5' -GAAGCTGCAGAATGGGATGACTTGCATCCAGTGCATGC-3' 5'-GCATGCACTGGATGCAAGTCATCCCATTCTGCAGCT TC-3'

MPD 028/029	MLV CA D83R	5'-CCG GGG CAA CCG TGG GCG CCC C-3' 5'-GGG GCG CCC ACG GTT GCC CCG G-3'
MPD 030/031	MLV CA R85D	5'-GGC AAC GAT GGG GAC CCC ACC CAA C-3' 5'-GTT GGG TGG GGT CCC CAT CGT TGC C-3'
MPD 045/046	MLV CA D83E	5'-GCTGTCCGGGGCAACGAGGGGCGCCCCACCC-3' 5'-GGGTGGGGCGCCCCTCGTTGCCCCGGACAGC-3'
GBM AU/AV	MLV CA R85K	5'-GCTGTCCGGGGCAACGATGGGAAGCCCACCCAACTGCCCAAC-3' 5'-GTTGGGCAGTTGGGTGGGCTTCCCATCGTTGCCCCGGACAGC-3'
GBM AQ/AR	MLV CA D83A	5'-GTCCGGGGCAACGCTGGGCGCCCCACCC-3' 5'-GGGTGGGGCGCCCAGCGTTGCCCCGGAC-3'
GBM AS/AT	MLV CA R85A	5'-GCTGTCCGGGGCAACGATGGGGCCCCCACCCAACTGCCCAAC-3' 5'-GTTGGGCAGTTGGGTGGGGGCCCCATCGTTGCCCCGGACAGC-3'

Table 2.1 Primers for site-directed mutagenesis

methylated template DNA. 5µl of this reaction was used to transform 50µl of XL-10 Gold Ultra-competent cells (Stratagene). Colonies were picked and DNA prepared. Plasmid integrity was verified by restriction enzyme digestion and the introduction of the correct mutation confirmed by DNA sequencing.

2.1.3 Agarose gel electrophoresis

In order to separate DNA by size for analysis and further purification, DNA was added to 6x DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll). Samples were loaded on 1% (w/v) agarose (Melford), 0.4µg/ml Ethidium Bromide, TBE (0.09M Tris, 0.09M borate, 2mM EDTA, pH8.4) gel and run at 100V. Smartladder size markers (Eurogentec) were also included.

2.1.4 Purification of DNA from agarose gels

To purify PCR products or bands from restriction enzyme digest, samples were separated by size by agarose gel electrophoresis. Bands were excised and the DNA extracted using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturers guidelines. The gel slice is dissolved in a solubilisation buffer at 50°C, then bound to a silica membrane in the presence of high salt. The sample is then washed followed by elution (50µl) of bound DNA in low salt.

2.1.5 Transformation of chemically competent bacteria

Chemically competent *E.coli* TOP10 cells (Invitrogen) and DNA (typically 50ul and 5µl) were incubated on ice for 30 minutes prior to heat shock at 42°C for 30 seconds. Cells were allowed to recover on ice for 2 minutes before incubation for 1 hour with 200µl prewarmed SOC media (20g bacto-tryptone, 5g bacto-yeast extract, 8.55mM NaCl, 2.5mM KCl, 10mM $MgCl_2$ and 20mM Glucose made up to 1 litre). Cells were then spread onto LB agar plates supplemented with the appropriate antibiotic (either ampicillin or kanamycin (Sigma) at 50µg/ml) and grown overnight at 37°C.
XL10 gold cells (Stratagene) were transformed in the case of site-mutagenesis reactions. In this case, 150µl cells were preincubated with 4µl β-mercaptoethanol for 10 minutes prior to the addition of DNA.

2.1.6 Purification of plasmid DNA from bacteria

Small scale purification by mini-prep.

Plasmid DNA from 1 ml bacterial cultures picked from single colonies was grown overnight in LB broth (10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl, in 1 litre, pH7.5) supplemented with an appropriate antibiotic (either ampicillin or kanamycin at 50μg/ml) using the Purelink Quick Plasmid Miniprep Kit (Invitrogen) according to the manufacturers protocol. Using this kit, cells are lysed by alkali/SDS, DNA bound to a silica membrane in high salt, washed then eluted in 50μl TE buffer.

Large scale purification by midi-prep.

Plasmid DNA was isolated from 100ml bacterial cultures, grown overnight in LB broth inoculated with 100μl of a mini-prep culture, using the Plasmid Midi Kit (Qiagen). Using this kit, cells are lysed by alkali lysis/SDS, the DNA bound to anion-exchange resin in low salt/pH conditions, washed in a medium salt buffer, then eluted in high salt buffer. DNA is precipitated by addition of isopropanol, allowing concentration and desalting by centrifugation and further washes. DNA is resuspended in TE buffer.

Purity and quantity of DNA.

DNA concentration and purity was determined using a spectrophotometer (Biophotometer, Eppendorf). Concentration is displayed automatically , though can be calculated on the basis that an absorbance of 1.0 at a wavelength of 260nM in a 1cm cell corresponds to 50μg/ml of double stranded DNA. Purity of DNA can be estimated by determining the A260:A280 ratio. A ratio of 1.8 or greater suggests that the DNA is relatively free from protein contamination.

2.1.6 Restriction enzyme digest of DNA

To confirm the identity of plasmids according to restriction digest profile or for sub-cloning, DNA was digested with restriction enzymes. All enzymes were supplied by Roche and used in buffers recommended by the manufacturer. A typical reaction consisted of 5ug of plasmid DNA, 2μl 10x reaction buffer, 1μl restriction enzyme

made up to 20µl with H_20, incubated at 37°C for 2 hours. Reactions were then analysed by agarose gel electrophoresis.

2.1.7 DNA ligation

Ligation reactions were carried about at 16°C for 16 hours using T4 DNA Ligase (New England Biolabs) in the buffer supplied. A typical reaction consisted of a 5:1 molar ratio of insert:vector, 2µl buffer and 1µl ligase made up to 20µl with H_20.

2.1.8 The gateway retroviral expression system

Retroviral Gateway Expression.

CFP-α-tubulin, GFP-Trim5α, and Trim5α were expressed in cells from a MLV based retroviral vector. Constructs were introduced into this vector using the gateway cloning system (Invitrogen). This system takes advantage of bacteriophage lamba site specific recombination. A PCR product is sub-cloned into pENTR-D-TOPO (Invitrogen). There it is flanked by *att L* and *att* R sites. A retroviral destination vector, pLgatewayIRESYFP/ pLgatewayIRESG418 also possesses two such site flanking the lethal *ccd*B gene. A recombination reaction occurs in the presence of LR Clonase enzyme swapping the flanked DNA sequence. Reactions are used to transform Top10 chemically competent *E.coli* cells (Invitrogen). Cells transformed with the original destination vector are selected against by the presence of the lethal *ccd*B gene. Successful recombinations remove this gene. Colonies are picked, grown overnight, plasmid DNA prepared and screened for the presence of the new insert by restriction digest. pLGFP-pLRhTrim5αIRESG418, pLGFP-HuTrim5αIRESG418, pLRhTrim5αIRESYFP and pLHuTrim5αIRESYFP were supplied and tested by Dr. Melvyn Yap. pLCFP-α-tubulinIRESG418 was produced by the author. Its construction is detailed below.

CFP-α-tubulin fusion protein

In order to produce a CFP-tubulin fusion protein, CFP was amplified from pECFP (Clontech) by PCR using the following primers: forward, 5' CACCATGGTGAGCAAGGGCGAGGA 3', reverse, 5' CTTGTACAGCTCGTCCATGC 3'. Tubulin was amplified from pEYFP-Tub

(Clontech) using forward, 5' GCATGGACGAGCTGTACAAG 3', reverse, 5' TGGATCCTTAGTATTCCTCT

The primers were designed with overlapping regions and so the two segments could be fused in a joining PCR reaction using the two original PCR products with the forward CFP primer and the reverse tubulin primer. The forward CFP primer was designed to commence with CACCATG allowing directional cloning into pENTR-D-TOPO. In this reacton, 4µl of gel purified PCR product was incubated for 5 minutes at room temperature with 1µl of the provided vector and 1µl of the provided salt solution. The entire mixture was then used to transform 50µl of TOP10 *E.coli* cells, selecting for resistance to kanamycin. Colonies were picked, grown overnight, DNA prepared and then screened for presence of the insert. An LR recombination reaction was then carried out to transfer the CFP-α-tubulin fusion into pLgatewayIRESG418. 75ng each of the entry clone and destination vector are combined with 1µl of LR Clonase reaction buffer, made up to 5µl with TE and incubated for and for one hour at 25°C. The reaction was stopped by addition of 1µl Proteinase K and incubation at 37°C for 10 minutes. The entire mixture was then used to transform 50µl *E.coli* cells, selecting for ampicillin. Colonies were picked, grown overnight, DNA prepared and screened for the presence of the insert by restriction digest.

2.1.9 DNA sequencing

DNA was sequenced using the BigDye Terminator cycle sequencing kit (Applied Biosciences) using the manufacturers protocol. 300ng DNA was included in a reaction with 3.2pmol of primer and BigDye reaction mix. Samples were denatured for 1 min at 96°C prior to 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min. Reactions were then precipitated by addition of 0.1 volumes of sodium acetate pH 5.2 and 2.5 volumes of 100% ethanol followed by 10mins incubation on ice. Samples were centrifuged at 16,000g for 10mins to pellet DNA. Samples were washed once with 70% ethanol. The complete reactions were analysed on a MegaBACE capillary sequencer (Pharmacia).

2.2 Cell culture and virus preparation

2.2.1 Cell culture

All cell lines were cultured in Dulbecco's modified Eagle medium (D-MEM) (Gibco) containing 10% heat inactivated fetal calf serum (Perbio) and antibiotics (100u/ml penicillin, 100µg/ml streptomycin, Sigma) 37° C in 5% CO_2. All cell lines used were adherent and so were maintained in tissue culture flasks (NUNC) In order to detach cells for passage and sub-culture, cells were washed with 1x Phosphate Buffered Saline (PBS) (10g NaCl, 250mg KCl, 1.437g Na_2HPO_4, KH_2PO_4 made up to IL with H_2O, pH7.4) prior to incubation with Trypsin-EDTA (0.05% (w/v) trypsin, 0.53mM EDTA).

2.2.2 Calcium phosphate transfection

Plasmid DNA was introduced into 293T cells by Calcium Phosphate Transfection using the Profection Mammalian Transfection System (Promega) according to the manufacturers protocol. Briefly: $2x10^6$ cells were plated 24hrs prior to transfection in a 6cm dish. Media was replaced 3 hours prior to transfection. 21µg DNA (made up of several plasmids depending upon the purpose of the transfection) was combined with 37µl Calcium Chloride and made up to 300µl with H_20. This was added drop wise to 300µl 2xHEPES-Buffered Saline (50mM HEPES, 280mM NaCl, 1.5mM Na_2HPO_4, pH 7.1). The mixture was incubated at room temperature for 30 minutes before adding drop wise to the cells. Cells were incubated overnight at 37°C. Media was replaced approximately 16 hours.

2.2.3 Virus preparation

Viruses were typically produced by $CaPO_4$ transfection of $4x10^6$ 293T cells with 7µg plasmid each of DNA encoding vector, Gag-Pol and Env components in 60mm dishes. For larger preparations, amounts of DNA were increased in proportion to the number of cells used. To enhance CMV driven promoter expression, media was replaced after approximately 16hrs post-transfection with fresh media containing 10mM sodium butyrate for 8-10hrs. After 48 hours, the virus containing supernatant

was harvested and filtered through a 0.45μM filter, allowing passage of virions but not cells. Virus was either used immediately or aliquoted and stored at -70°C.

All viruses were pseudotyped with the VSV-G Env protein expressed from pczVSV-G (Pietschmann et al., 1999). MLV Gag-Pol was expressed from either pHIT 60 (NB-tropic) pCIG-N (N-tropic) or pCIG-B (B-tropic) or variants produced by mutagenesis thereof (Soneoka et al., 1995). HIV Gag-Pol was expressed from p8.91 or mutant variants (Naldini et al., 1996). Vectors varied dependent upon application. For HIV, pCSGW was used to deliver eGFP for flow cytometry and Q-PCR (Bainbridge et al., 2001). For MLV, pLNCG was used for eGFP for flow cytometry (Yap et al., 2004); pHIT 111 for LacZ in abrogation experiments; pLIB-NCA/BCA-puro for CA expression (see below); p583-gateway-iYFP/G418 (see cloning) for expression of various Trim proteins or pFv1NiYFP/pFv1BiYFP for Fv1 (Bock et al., 2000).

In some cases, infectious N-tropic MLV was harvested from a *M.dunni* cell line chronically infected with the virus clone WN41 (A. Stevens, unpublished data). In these cases, cells were grown to confluency in a 15cm dish with 25ml of media. Cells were incubated for 2 days and after that time, the virus containing supernatant was harvested and filtered.

2.2.4 Transduction of cells and flow cytometry

Determining infectivity of virus

In order to examine the infectivity of mutant viruses, $4x10^4$ cells were split into wells of a 12-well dish (*M.dunni* for MLV, TE671 for HIV-1) 16 hours prior to transduction. Increasing amounts of virus carrying an eGFP vector was added to the cells. 2 days later, cells were harvested an analysed by flow cytometry.

Producing multiply transduced stable cells

To stably express proteins in cells, MLV based vectors were packaged using the NB-tropic pHIT60 packaging plasmid and were used to infect $4x10^4$ cells at a multiplicity of infection of around 10 (500μl of virus added) Typically 99.9% of cells are transduced. This was confirmed by flow cytometry or fluorescence microscopy after expansion and culture for 2 weeks.

Flow cytometry

Cells were harvested 2 days post transduction by trypsinisation and fixed in PBS-4% paraformaldetype (pH 7.4). The percentage of fluorescence positive cells (typically eGFP or YFP) was determined using a FACS Calibur (Becton Dickenson) and data was analysed using Flow Jo software (Tree Star Inc.)

2.3 SDS polyacrlamide gel electrophoresis (SDS-PAGE) and western Blot

2.3.1 SDS/PAGE

Denaturating 10% or 12% poly-acrylamide gels were cast using the mini-PROTEAN II cell system (BIO-RAD) with 1.5mm spacers. Running gels were made up to 10 or 12% acrylamide (stock 30% Acrlamide/Bis 37.5:1) (BIORAD), 375mM Tris-HCl, pH 8.8, 0.1% SDS, 0.1% ammonium persulpate (APS) , and 8µl TEMED. 2.5ml stacking gels consisted of 5% Acrylamide, 124mM Tris-HCl, pH6.8, 0.1% APS, 0.1% and 10µl TEMED.

Samples were resuspended in 1X (or diluted 5X) SDS loading buffer (1X: 10% Glycerol, 2% SDS (w/v), 60mM Tris, pH6.8, 0.001% Bromophenol blue, 100mM DTT and heated to 100°C for 5 minutes. Gels were run at 20mA for 1-2 hours in running buffer (0.1% SDS, 27.6mM Tris, 0.2M Glycine, pH 8.8).

A 10-170kDa pre-stained protein ladder (Page ruler, Fermentas) was used as a standard.

2.3.2 Transfer to PVDF membrane and western Blot

Protein separated by SDS-PAGE was transferred to PVDF membrane (Immobilon – P, Millpore) using semi-dry transfer apparatus (BIORAD). Membrane was prepared by wetting for 1 minute in methanol followed by 15 minutes in transfer buffer (20% Methanol, 39mM Glycine, 0.037% (v/v) SDS, 48mM Tris). Transfer was typically 1 hour at 20 volts. The membrane was blocked overnight at 4°C in 5% non-fat dry milk (Marvel), PBS 0.1% polyoxyethylene-sorbitan monolaurate (TWEEN 20)(Sigma). Membranes were probed with the appropriate antibodies at the concentrations shown in table for 1 hour. Membranes were washed 3 times in PBS, 0.5% TWEEN 20 prior

Target / Purpose	Type	Manufacturer / Reference	Dilution WB	Dilution IFA	Other
MLV capsid	Rat Monoclonal	ATCC CRL-1912 (Chesebro et al., 1983)	1:10000	1:1000	Also used for IP
Biotinylated MLV CA	Goat polyclonal	Viromed	N/A	N/A	Used in ELISA
MLV p12	Goat polyclonal	Viromed	1:10000		
MLV p12	Mouse Monoclonal	ATCC CRL1890 (Chesebro et al., 1983)	1:10000	1:1000	
HIV capsid (p24)	Goat polyclonal	ARP 322	1:1000		
HIV capsid	Mouse monoclonal	Malim Group 24-2	1:1000		
Trim5α	Goat polyclonal	Abcam	1:1000		
Alpha tubulin	Mouse monoclonal	Sigma	1:10000		
Cyclophilin A	Rabbit polyclonal	Biomol	1:500		
Anti-Goat HRP	Horse polyclonal	Vector Laboratories	1:2000		
Anti-Rabbit HRP	Goat polyclonal	Pierce	1:5000		
Anti-Mouse HRP	Rabbit polyclonal	Pierce	1:2000		
Anti-Rat HRP	Goat polyclonal	Pierce	1:1000		
Alexafluor 488 Anti Rat	Goat polyclonal	Molecular Probes		1:400	
FITC Anti Mouse	Goat polyclonal	Santa Cruz Biotechnology		1:400	

Table 2.2 Antibodies used in this study

to incubation with a horseradish peroxidase conjugated secondary antibody. The membrane was again washed 3 times. The ECL Chemiluminescence kit (Amersham), was used to detect bound secondary antibody by exposing X-ray film (Fotochemische Werke GMBH, Berlin).

A list of all antibodies used and their appropriate dilution is shown in table 2.2.

2.3.3 Analysis of virus preparations and cell extracts by western blot

Virus preparations

500µl of filtered virus containing 293T supernatant was centrifuged through a 20% sucrose (in PBS) cushion at 100,000g for one hour. The supernatant was then removed and the pellet resuspended in 100µl SDS/PAGE loading buffer. Typically, 10µl was then subjected to western blotting as described above.

Cell extracts

After removal of virus containing supernatant, the 293T cells used for virus production were washed twice with PBS. Cells were then lysed using a triple detergent lysis buffer (50mM Tris.Cl (pH 8.0), 150mM NaCl, 0.02% sodium azide, 0.1% SDS, 100µg/ml phenylmethlysulfonyl fluoride (PMSF), 1µg/ml aprotinin, 1% NP-40, 0.5% sodium deoxycholate) (Sambrook et al., 1989) supplemented with EDTA free protease inhibitor cocktail (Roche). After lysis for 10 minutes at 4°C, lysates were cleared by centrifugation at 16,000g for 30 minutes. Protein content was determined by Bradford assay and 15µg was subjected to western blotting as described above.

In the experiments used to examine Trim5α expression levels in cells, cells were grown to confluency in 6cm dishes then lysed in 500µl triple detergent lysis buffer. Extracts were processed as above. 50µg of protein was loaded per lane.

Bradford assay

Assays to determine total protein content of cell extracts were carried out with the Bio-rad Protein Assay kit according to the manufacturers guidelines. Standards were prepared using bovine serum albumin (BSA) (Sigma) at 1mg/ml, 0.75mg/ml. 0.5mg/ml. 0.25mg/ml, and 0.1mg/ml. The reagent was diluted 1 in 5 in H_20. 5µl of standard or sample was added to 500µl of diluted reagent in a 1cm cuvette. After 5

minutes, absorbance was measured using a spectrophotometer and a standard curve plotted. Concentration of protein in the sample could then be calculated from the equation of this line. Cell extracts were typically in the range of 5-7.5mg/ml and so were diluted 1 in 10 prior to testing.

2. 3 Abrogation experiments

In all cases, cells were plated at a density of $4x10^4$ per well in 12-well plates 24hrs prior to transduction. In the case of the MLV experiments described in Chapter 3, cells were pre-treated with 500µl of the appropriate virus (abrogating particle, AP) (Lac Z vector) at the concentrations described in figure legends, for 3 hours, before challenge with a fixed amount of either N or B tropic virus carrying the pLNCG (eGFP) vector. In the case of HIV experiments described in Chapter 6, cells were pretreated with the indicated volume of virus preparation made up to 500µl with fresh media. The was followed by challenge 3 hours later with a fixed dose of HIV carrying an eGFP vector. The percentage of transduced cells was analysed by flow cytometry after 3 days.

2.4 Derivation of single cell clones expressing CA

To insert a foot and mouth disease virus 2A protease between p12 and CA, thereby allowing intracellular expression of properly processed CA the following procedure was employed. The recombinant DNA aspect of this procedure was carried out by Dr. Michael Bock and is included here for completeness. The *gag* region of pCIG3 N and B was amplified by PCR in two sections. Primers were designed to produce a *EcoR1*-MA-p12-(N term 2A)-*Hind III* PCR product and a *Hind III*–(C term 2A)-CA-*Mfe I* PCR product. The following primer sets, were used:
MA-p12-2A, fwd, 5'-GCGAATTCACATGGGACAGACCGTAACC-3', reverse 5'-GCAAGCTTAAGAAGGTCAAAATTCAACAGAAAGCCCCGAGAGGTGGTGG-3';2A-CA,forward,
5'GCAAGCTTGCGGGAGACGTCGAGTCCAACCCCGGGCCACTCCGTTTG-3', reverse, 5'-CGCAATTGTTACAAAAGTTTGCTCATTTCTCTA-3'.

Each product was cloned into pCRII-TOPO (Invitrogen). These plasmids were then digested with either *EcoRI/Hind III* for the MA-p12-2A insert or *Hind III/Mfe I* for the 2A-CA insert. A triple ligation was performed with these two inserts and the pIRESpuro vector (Clontech) (previously cut with *EcoRI*), creating MA-p12-2A-CA-IRES-puro. The full construct was excised with *EcoRI* and *StuI* and ligated into pLIB (Clontech), previously cut with the same enzymes, producing pLIB-NCA-puro and pLIB-BCA-puro.

Viral vectors carrying pLIB-NCA-puro and pLIB-BCA-puro were prepared following transfection of 293T cells. Serial dilutions were prepared of each virus (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}), with 100µl of each used to infect a 6cm plate with $5x10^5$ NIH-3T3, Balb-3T3, *M. dunni*, or TE671 cells. After 2 days, puromycin (Sigma) was added to the media at a concentration of 2µg/ml.

Two weeks later plates with separate colonies could be identified for each cell line and expression construct. Three colonies of each type were isolated with cloning rings (Sigma) and grown as individual cultures. Cells were screened for CA expression by western blot analysis.

2.5 MLV CA ELISA

2.5.1 Capture antibody preparation

IgG fractions from 3ml of goat anti-CA polyclonal antisera (Viromed) were purified on a protein A column as described by Harlow (Harlow and Lane, 1988). The pH of the sera as adjusted to pH 8.0 by addition of 1/10 volume of 1M Tris (pH8.0). The the sera was then passed through a 1ml protein A bead column. The column was then washed with 10 volumes of 100mM Tris (pH 8.0) follow by 10 further volumes of 10mM Tris (pH 8.0). The antibody was eluted by stepwise addition of 250µl 100mM glycine (pH 3.0). Protein concentration was determined by Bradford assay of the fractions. Fractions 7-12 give concentrations greater than 10mg/ml and so were pooled. This material was dialysed overnight into 0.1M Sodium Phosphate (pH 7.2) prior to biotinylation.

The purifed antibody was biotinylated using the Immunoprobe Biotinylation Kit (Sigma) according to the manufacturers guidelines. Briefly, 1ml of antibody

preparation at 10mg/ml was combined with the biotinylation reagent BAC-SulfoNHS. The labelled antibody is separated from the unreated reagent by gel-filtration using a Sephadex G-25 column provided. Column fractions containing protein were pooled resulting in a 3.8mg/ml preparation of biotinylated antibody.

2.5.2 ELISA protocol

Wells from a 96 well Reacti-Bind Streptavidin Plate (Pierce) were washed 3 times with 250µl PBS; 0.1% BSA; 0.05% Tween before being incubated overnight at 4°C with 100µl of 25µg/ml biotinylated anti-CA (diluted in wash buffer). Plates were washed 3 more times before the addition of 100µl of viral sample, pre-treated with 1% NP-40 for 15 min at 37°C to disrupt viral membranes, then incubated at room temperature for one hour. After 3 further washes, rat monoclonal anti-CA antibody (100µl) was then added at a concentration of 10µg/ml (diluted in wash buffer) and the plates were incubated for a further 90 min at room temperature. After 3 more washes, 100µl of monoclonal goat anti-rat IgG conjugated to calf intestinal phosphatase (Pierce) was added at a concentration of 6µg/ml and incubated at room temperature for one hour. The wells were then washed 10 times with wash buffer. Binding of this antibody was detected using the Phosphatase Substrate Kit (Pierce) according to the manufacturers protocol. A405 was measured using a Thermo Labsystems Multiskan Ascent spectrophotometer. Concentrations were determined from the mean A405 of 3 replicates. Standard curves were obtained with N-terminal his-tagged CA purified from *E.coli* generously supplied by Dr. Nehar Mortuza. (Mortuza et al., 2004).

2.6 Immunoprecipitation and finger printing by MALDI mass spectrometry

M. dunni cells expressing CA from N tropic MLV were lysed using a non-denaturing lysis buffer and the CA immunoprecipitated using rat monoclonal anti-CA antibody bound to protein G beads as described by Coligan(Coligan, 1996). Beads were then resuspended in SDS/loading dye containing 10mM DTT and then incubated at 90°C for 5 minutes to detach the bound protein. Following centrifugation, the supernatant

was loaded onto a 12% SDS/PAGE gel. After electrophoresis, gels were stained with Simply Blue Safe Stain colloidal coomassie (Invitrogen) and the band at 30kDa excised, reduced with dithiothreitol and alkylated using iodoacetamide. Gel slices were dried and reswollen in a sufficient volume to cover of 4ng/µl Endoproteinase Asp-N (sequencing grade, Roche) in 5mM ammonium bicarbonate. After overnight digestion at 32°C the supernatant was acidified by the addition of a 1/10th volume of 4% trifluoroacetic acid. Peptide mass fingerprinting was performed using a Reflex III MALDI time-of-flight mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany), equipped with a nitrogen laser and a Scout-384 probe, to obtain positive ion mass spectra of digested protein with pulsed ion extraction in reflectron mode. An accelerating voltage of 26kV was used with detector bias gating set to 2kV and a mass cut-off of m/z 650. Matrix surfaces were prepared using recrystallised α-cyano-4-hydroxycinammic acid and nitrocellulose using the fast evaporation method (Vorm and Roepstorff, 1994). 0.4µl of digestion supernatant was deposited on the matrix surface and allowed to dry prior to desalting with water. Peptide mass fingerprints were compared against the non-redundant protein database placed in the public domain by the National Centre for Biotechnology Information (NCBI) using the program MASCOT (Perkins et al., 1999). Mass spectrometry was carried out by Dr. Stephen Howell.

2.7 Immunofluoresence

Cells were plated at a density of $2x10^4$ per well on a glass cover slip in a 12 well plate 24 hours prior to staining. Cells were washed with PBS then fixed for 10 minutes at room temperature in 4% paraformaldehyde. To allow entry of antibodies, cells were permeablised with 0.2% Triton X in PBS for 10 minutes at room temperature prior to blocking with 1% BSA in PBS (PBS/BSA) for 15 minutes. Cells were then probed with the appropriate antibody diluted in PBS/BSA. After 1 hour incubation at room temperature, cells were washed three times for 5 minutes each with PBS/BSA followed by incubation with secondary antibody (in PBS/BSA) for 30 minutes. This was followed by detection with a fluorescent conjugated secondary antibody. Coverslips were washed 3 more times with PBS, placed cell side down in glycerol on slides and sealed using nail varnish. Slides were viewed using a

Deltavison Olympus IX70 inverted microscope with Softworx image acquisition software through a 100x objective lens.
Antibodies used and their concentrations are shown in table 2.2.

2.8 Live cell microscopy, image processing and analysis

2.8.1 Live cell microscopy

Human TE671 cells were transduced with the appropriate expression construct at an m.o.i. of approximately 10 using a Moloney MLV vector expressing CFP-α-tubulin (pLCFP-α-tubulinIRESG418), or GFP-Trim5α from either human or rhesus macaque pLGFPTrim5αRhIRESG418, pLGFPTrim5αHuIRESG418) After 2 weeks of culture, $4x10^4$ cells were plated in Mattek 35mm Glass Bottom microwell dishes 24 hours prior to microscopy, then viewed using a Deltavision Olympus IX70 inverted microscope through a 100x objective lens with Softworx image acquisition software. Cells were incubated at 37°C during microscopy in the presence of CO_2 independent media (Invitrogen). Images were captured either indivually or in sequence as described in *Results*.

2.8.2 Image processing and analysis

The data captured in movie files was extracted from the microscope software as a series of individual tif files. These were converted to bmp format using with the image processing software, Image J. The adjustments to brightness and contrast described in figure 7.7 following geldanamycin treatment were made using Image J.
Image sequences were combined into a bmp stack allowing the data to once again be stored and handled as one file.
In order to represented particle movement data in a printed format as shown in figure 7.2, 60 frames were added to form one image using Image J. Images were summed on the basis that in the composite image, any given pixel is assigned the maximum intensity value observed throughout any of the 60 input images.

2.8.3 GMview

In order to analyse the data within these images sequences, the particle tracking software GMview (Gregory Mashanov, Division of Strutural Biology, NIMR) was employed in two ways. Data is imported into the GMview software as a sequence of bmp images.

The first technique allowed manual tracking of user defined particles by clicking the computer mouse on the particle to be tracked in each frame. The software reveals data described in figure 7.3 showing the average velocity and velocity between frames.

The second process used the automatic particle tracking function of GMview. In these circumstances, all fluorescent objects within each frame are tagged by the software. Particles above a user defined size and intensity (altered dependent upon the background signal in the individual sample) are tracked between frames. Tracks are made between nearest neighbours in consecutive frames. A minimum length of tracking of 5 frames was specified and a maximum range of movement defined as 12 pixels, corresponding to 1.632μm in all of the cells examined. The potentially allows for movement rates of up to 1.23μm/second to be followed. After the automatic analysis, each track can be manually verified as authentic. This technique was used to determine the effect of nocodazole on motility of cytoplasmic bodies. Cells were treated with 66μM nocodazole (Sigma) for two hours prior to microscopy by replacement of normal growth media with CO_2 independent media supplemented with the drug.Image sequences of 60 frames were acquired for 5 cells both treated and mock treated. Data from such an analysis is shown in figure 7.4.

2.9 Core preparations

30ml of virus containing supernatant from a 15cm dish of 293T cells transfected with pVSV-G, p8.91 and pCSGW for HIV or pVSV-G, pCIG-N and pHIT111 for MLV were passed through a 0.45μm filter then centrifuged for 2 hours at 100,000g through a cushion of 20% (w/v) sucrose in STE buffer (10mM Tris, 100mM NaCl, 1mM EDTA) using a Beckman SW-28, rotor to concentrate the virus. Pelleted virions were gently re-suspended in 250μl STE buffer by shaking at 4°C for 1 hour.

A 10ml 30-70% continuous sucrose gradient in STE buffer was prepared using a gradient maker (Minipuls 2, Gilson). On the top of this, was layered 250µl of 1% Triton-X in 15% sucrose. This was followed by a 250µl layer of 7.5% sucrose in STE buffer. Above this, 250µl of concentrated virus was carefully layered. Gradients were centrifuged for 16hrs at 100000g at 4°C using a Beckman SW-41 rotor. Fractions of sizes indicated in figures were harvested after 16hrs from the bottom of the tube and their CA content analysed using an HIV CA ELISA (Innogenetics) or western blot in the case of MLV. Sucrose density was determined from the refractive index of the fractions using a refractometer. CA containing fractions of appropriate density to contain cores were pooled, aliquoted and stored at -70°C. During the course of these studies it became apparent that CA material could be lost via pipette tips and microfuge tubes (described in detail in Chapter 5), cores were handled from this stage onwards with pipette tips which had been blocked with 1mg/ml BSA then air dried.

2.10 Restriction factor binding assay

2.10.1 Cell extract preparation

Cells extracts containing restriction factor were prepared by freeze-thaw lysis of TE671 cells stably transduced with Trim5α of Rhesus Macaque origin. 2x10cm dishes of confluent cells were washed twice in STE buffer supplemented with protease inhibitor cocktail (STE/PI) (Roche). The dish was tilted to allow all of the wash to accumulate and be removed by pipette. Cells were removed by scraping using a cell scraper (Corning). Cells were collected in a microfuge tube then subjected to 2 freeze thaw cycles in liquid nitrogen. Examination by light microscopy typically revealed 90% lysis. Debris were removed by centrifugation at 100,000g for 30 minutes. The protein content of the cleared cell extract was determined by Bradford assay and was typically in the range of 5-7.5mg/ml. The extract was standardised at 2.5mg/ml by addition of STE/PI in all experiments.
A wash was prepared in a similar manner using non-transduced cells.

2.10.2 Binding assay

100µl of HIV core material (1000ng/ml in a typical preparation) was incubated with 300µl of cell extract prepared as described above for 1 hour at 4°C. The reaction was subjected to centrifugation at 16,000g for 30mins to pellet core material and any Trim5α associated with it. The supernatant was removed and the pellet washed with 500µl of wash prepared from a non-transduced cell extract. The sample was centrifuged again for 10mins at 16,000g. The wash was removed and the sample resuspended in 100µl SDS/PAGE loading buffer. 50µl of both pellet and supernatant and the original cell extract were analysed by western blot for Trim5α and HIV CA. Where ATP was presence, it was included in the incubation and wash at a concentration of 5mM, diluting from a 1M stock (pH 7)

2.11 HIV core disassembly assay

2.11.1 Disassembly assay

Core disassembly assays were based upon those described by Forshey. (Forshey et al., 2002). Cell extracts were prepared as described above.

45µl of HIV core material (1000ng/ml in a typical preparation) was diluted with the 415µl of the appropriate cell extract/buffer and incubated at 37°C. At the indicated time points, 150µl of the reaction was removed and subjected to centrifugation at 100,000g for 30 minutes to pellet assembled HIV core material. The supernatant was removed and the pellet re-suspended in 150µl STE/BSA . The amount of material in the pellet and supernatant was determined by HIV CA ELISA and the percentage disassembly calculated as amount in supernatant / (amount in supernatant + amount in pellet). Percentage recovery could also be calculated by comparing the amount in pellet + the amount in supernatant with input levels.

In the cases where the incubation was separated again on a sucrose gradient, a 10ml 70%-30% gradient was prepared as described above and the cell extract layered above. This was subjected to centrifugation at 100,000g for 16 hours at 4°C prior to analysis by HIV CA ELISA.

2.11.2 Fractionated cell extracts

In several experiments, fractionated cell extracts were used in incubations. In these cases, the extracts were passed through centrifuge concentrators (Vivascience) at 6,000g with specific molecular weight ranges (5kDa, 10kDa or 50kDa). The filtrate was used in the disassembly assay described above.

2.12 Analysis of intracellular CA in infected cells

$6x10^6$ TE671 or *M.dunni* cells either wildtype or heavily transduced with and stably expressing RhTrim5α or Fv1 were split into 80cm^2 flasks, 24 hours prior to infection. Virus was prepared as described earlier (VSV-G pseudotyped HIV-1 by transfection or N-MLV from a chronically infected line) and 10ml added to the flask. Infected cells were incubated for 16 hours at 37°C. After 16 hours, cells were washed 3 times with PBS before detachment with 1ml trypsin. 5mls DMEM was added to aid in removal of cells from the flask. Cells were centrifuged for 5 minutes at 200g to pellet. The pellet was washed twice with PBS then resuspended in 2mls hypotonic lysis buffer (10mM Tris-HCl (pH 8.0), 10mM KCL, 1mM EDTA) and incubated on ice for 15 minutes. The cells were lysed by using a 5ml Dounce homogenizer and 20 strokes. Cell debris was removed by centrifugation for 3 minutes at 2,000g. 100µl of the supernatant was taken for analysus. 2 ml of lysate was layered onto a 7ml 50% sucrose cushion (made in PBS) and centrifuged at 125,000g for 2 h at 4°C in a Beckman SW41 rotor. After centrifugation, 100µl from the top-most part of the supernatant was collected and prepared for western blot. The pellet was resuspended in 100µl of 1x SDS sample buffer. The samples were subjected to SDS/PAGE and Western blotting for capsid proteins.

2.13 Quantitative PCR

2.13.1 Following infection of cells

VSV-G psedotyped virus with a genome derived from pCSGW was prepared by co-transfection with wildtype or mutant Gag-Pol packaging plasmids. Virus stocks were treated with RQ1 DNase (Promega) at 10 units/ml for 1 h at room temperature to remove any DNA that could be carried over from transfection during viral production. $2.5x10^5$ TE671 cells were infected with 50µl of DNase treated virus. DNA was extracted from the infected cells after 7 hours using the DNeasy tissue kit (QIAGEN). Quantitative PCR for detection of early reverse transcription (strong-stop DNA) products was performed using the ABI Prism 7000 sequence detection system from Applied Biosystems with 150 ng of total DNA, 70 nM of each primer, and 1x SYBR Green mix (ABgene) in a reaction volume of 25 µl. The primers used were HIVEF 5' TCTGGCTAACTAGGGAACCCA 3' , HIVEL 5' CTGACTAAAAGGGTCTGAGG 3'. The program consisted of an initial incubation at 50°C for 2 min, followed by 95°C for 15 min before 40 cycles of 95°C for 15 seconds and 60°C for 1 min

2.13.2 Intravirion endogenous reverse transcripton (ERT)

ERT experiments were based upon those carried out by Powell and colleagues (Khan et al., 2001). 35µl of virus containing cell supernatant (as above) was incubated with 2.5mM $MgCl_2$, 30µM Spermidine, 5mM dNTPs (replaced by water in control) and 0.02% NP-40. Reactions were incubated at either 37°C or 4°C (control) for 4 hours. After 4 hours, DNase I (Promega) was added and reactions incubated at 37°C for 15 min to digest any extra-virion DNA. DNase was then inactivated by addition of 20mM EGTA and incubation at 65°C for 15 minutes. DNA was purified using the Qiamp DNA Mini Kit (Qiagen), and eluted in 200µl. The DNA was then incubated with DpnI for one hour. DNA was quantified by Q-PCR using DNA as described above using 5µl per reaction. Amount of CA in the original virus preparation was quantified by HIV CA ELISA.

2.14 Geldanamycin experiments

Cells were treated with geldanamycin (Invivogen) at the concentration and for the times indicated by replacement of media with that containing geldanamycin diluted from a 1mM stock.

2.15 Electron microscopy

2.15.1 Thin section EM of cells transduced with Gag-Pol plasmids

6cm dishes were transfected with 21μg of Gag-pol plasmid as described earlier and the protocol of virus production followed. After 2 days, cells were washed and fixed in 2% glutaraldehyde/2% paraformaldehyde in 0.1M sodium cacodylare buffer pH 7.2 for 1 hour scraped and pelleted, left in the same fixative overnight then treated as described below. In the case of virus preparations, transfected cell supernatants were adjusted to 20% FCS, 2% glutaraldehyde, 2% paraformaldehyde, 0.1M sodium cacodylate (pH 7.2). Samples were centrifuged for 90 minutes at 13,000g to pellet virions and fixed in 100μl 2% glutaraldehyde, 2% paraformaldehyde, 0.1M sodium cacodylate overnight. Pellets were then over-layed with 2% low melting point agarose in 0.1M sodium cacodylate buffer.
Both cell and viral pellets were then treated as follows. Samples were washed 10 min and post fixed in 1% osmium tetroxide in the same buffer for 1.5 hours. They were washed again and stained en bloc with 1% aqueous uranyl acetate for 1.5 hours, then dehydrated in a graded ethanol series 50%, 75% 90% and 3x100% for 10 mins each and then propylene oxide 2x 15mins. They were embedded in medium Agar 100 resin (Agar 100 20 ml, DDSA 16 ml, MNA 8 ml, BDMA 1.3 ml) 24 hours then polymerized at 70°C overnight. 50μm sections were mounted on pioloform coated slot grids and stained with saturated ethanolic uranyl acetate followed by Reynold's lead citrate. They were viewed in a Jeol 1200EX TEM.
Preparation of samples after fixation was carried out by L. Hirst. Microscopy was performed by the author.

2.15.2 Negative stain EM of purified virus cores

3 ml of virus core material was diluted with 10 ml STE buffer. The sample was centrifuged at 100,000g for 1 hour and the pellet resuspended 50µl STE buffer. 50µl of 4% paraformaldehyde was added to fix the sample. Samples were absorbed onto a carbon coated grid and negatively stained with 1% sodium silicotungstate (pH7.5). A JEOL 1200EX electron microscope operated at 100KV was used to view the grids. Micrographs were taken under minimum dose conditions..

2.16 Molecular representations

All molecular representations were produced using the software package Pymol using co-ordinates obtained from the Worldwide Protein Data Bank (PDB).

CHAPTER 3

Requirements for Gag Processing and Core Formation in the Abrogation of Fv1 and Trim5α restriction of Murine Leukemia Virus

Restriction by both Fv1 in mouse cell lines and Trim5α in human cells is saturable. Challenge by virus at a high multiplicity of infection can overcome both blocks leading to successful infection of the target cells.

Furthermore, it is possible pre-treat cells with a large quantity of restricted virus, overcome the restriction factor, then subsequently challenge the same cells several hours later with a second restricted virus. This second virus will now successfully infect the cells. This process is known as abrogation. If this second virus carries a fluorescent marker within its genome such as GFP, then its successful integration and subsequent transcription and translation of the fluorophore can be detected by flow cytometry (Duran-Troise et al., 1977; Towers et al., 2002).

Given the difficulties already described in studying the direct interaction between Fv1 and the MLV CA protein, abrogation offers a useful, if somewhat indirect, method to study the interaction between virus and restriction factor. Mutations can be introduced into the Gag proteins of virions used to pre-treat cells (hereafter referred to as *abrogating particles, APs*) and their ability to abrogate restriction determined. Conclusions can therefore be drawn about their ability to interact with the restriction factor.

After release from the producer cell, the viral protease cleaves the MLV Gag poly-protein into its four constituent parts, leading to the condensation of the CA component to form a multimeric spherical core containing the RNA genome. This chapter describes experiments based upon abrogation, designed to address possible involvement of Gag processing and CA core maturation in the interaction of Fv1 and Trim5α with the virus CA protein using a series of mutants defective for these processes.

3.1 Determination of the amount of virus required for abrogation

Before beginning experiments to test the ability of Gag processing mutants to abrogate restriction, it was important to characterise properly the quantity of wildtype virus required for saturation of restriction in the cell lines to be used.

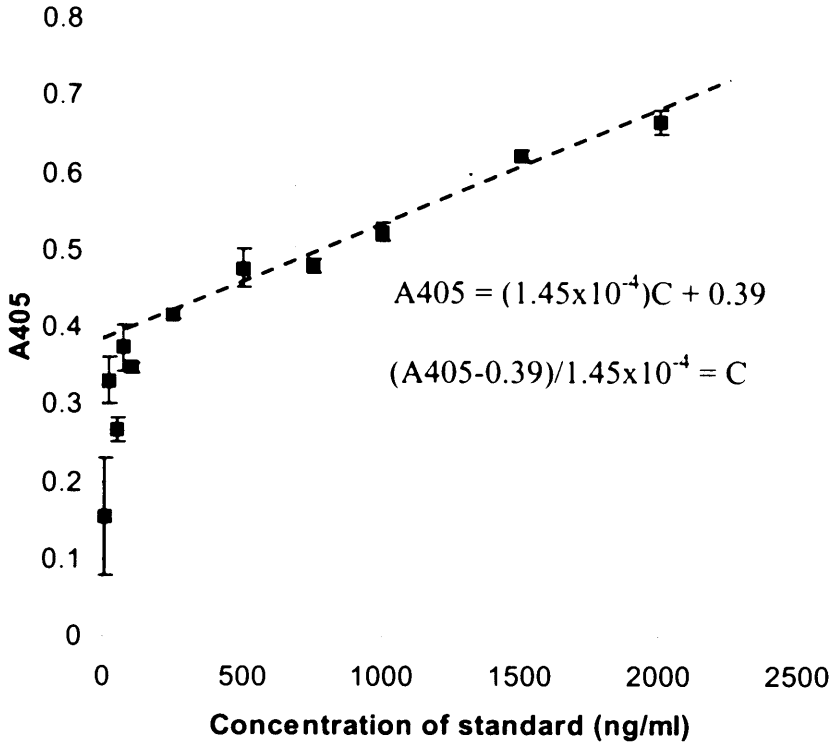
3.1.1 An enzyme-linked immunosorbent assay (ELISA) to quantify MLV capsid protein in producer cell supernatant

Genetic evidence strongly implicates the MLV CA protein as the target for both Fv1 and Trim5α. It was therefore decided to quantify MLV based upon the amount of CA present in virions, rather than any other viral component such as RT as it is conceivable that its relative incorporation into virus particles may vary when mutations are made in Gag (Tang et al., 2003). An ELISA based system was chosen because it allows rapid and simultaneous processing of a large number of samples.
Procedural details of the assay are described in *Materials and Methods*. Affinity purified recombinant N-terminal domain MLV CA was used to generate a standard curve. This curve reproducibly showed a linear relationship between standard concentration and absorbance at 405nm over a range of approximately 500-2000ng/ml. A405 values obtained when viral supernatant was used in this system typically fell within this range when used at either a 1:1 or 1:3 dilution, again with a linear relationship between virus added and A405 (Figure 3.1)
Although there is no data on absolute levels of MLV CA produced using the 293T three plasmid transfection system, when HIV is produced in a similar fashion and measured using commercial CA ELISA kits, values obtained are comparable. This assay therefore appears to be a good method to determine both absolute and relative levels of CA in viral supernatants.

3.1.2 Abrogation properties of mouse and human cell lines

Human TE671 cells (expressing Trim5α and therefore restricting N-tropic MLV), as well as murine Balb/3T3 (expressing $Fv1^b$, restricting mainly N-MLV) and NIH 3T3 (expressing $Fv1^n$ and restricting B-MLV) cells were pre-treated with increasing

A



B

Ratio virus: diluent	A405 (mean of 3 measurements)	Calculated CA concentration (ng/ml)
Non diluted	0.801	2834
1:1	0.581	1317
1:3	0.492	703

Figure 3.1 An enzyme linked immunosorbent assay to measure absolute amount of MLV CA in producer cell supernatant.
(a) As described in materials and methods, the purified recombinant N-terminal domain of CA, of known concentration was used to generate a standard curve. The linear region of this curve was identified and the relationship between concentration and A405 was determined using the equation y= mx +c where m is the gradient and c is the y-intercept. The resulting equation can then be rearranged to define concentrations of CA (C) in terms of A405.
(b) Filtered 293T Supernatant produced from a typical transfection to produce MLV was used in the assay either neat, at 50% dilution or at 25% dilution and the A405 determined. The apparent CA concentration was then determined based upon the standard curve.

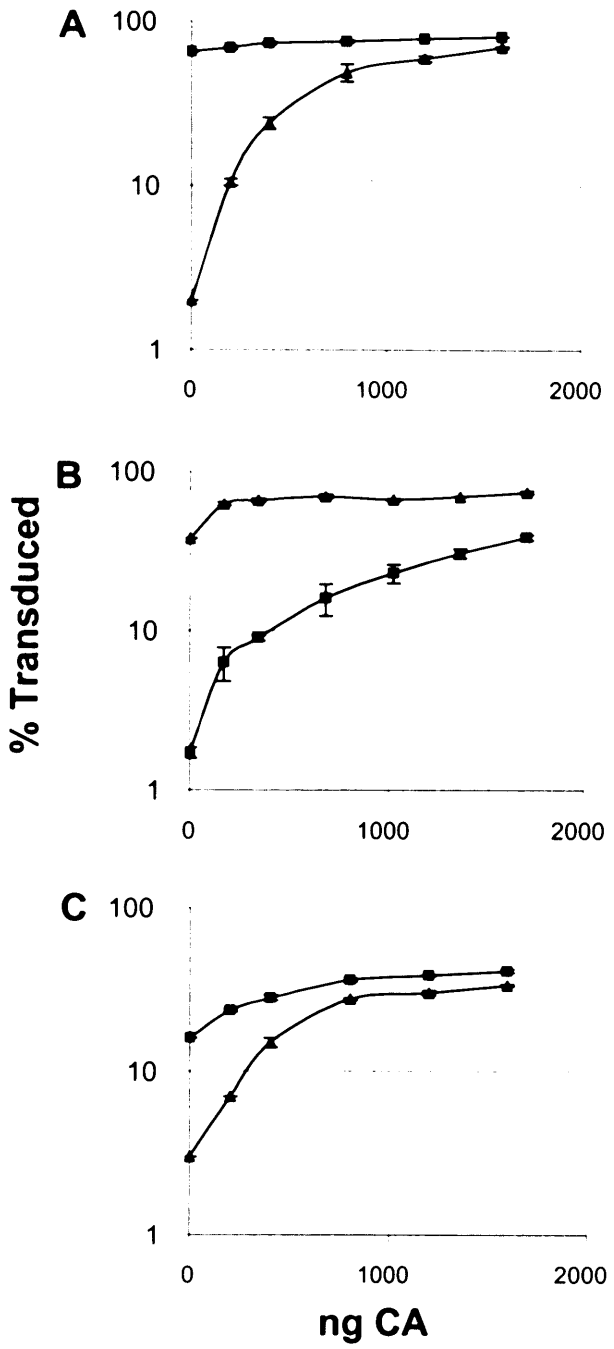


Figure 3.2 Abrogation Curves for Trim5α and Fv1 Expressing Cells.
$4x10^4$ cells were pre-treated with increasing amounts of N-tropic VLP (TE671 (a) and Balb/ 3T3 (c)) or B-tropic VLP (NIH 3T3 (b)) as determined by MLV CA ELISA. Cells were then infected with an equal amount of N (-▲-) or B (-■-) MLV carrying the eGFP vector. The percentage of transduced cells was determined by flow cytometry. Data shown are the mean of three experiments. Error bars show standard deviation.

amounts of restricted virus and then challenged with equal amounts of N-tropic or B-tropic virus carrying a GFP vector. The percentage of cells transduced was determined by flow cytometry and plotted against the amount of CA used for abrogation as determined by ELISA. Significant differences were seen between the cell lines (Figure 3.2).

As expected, treatment of TE671 cells with N-tropic APs had little or no effect on infectivity of B-tropic virus, but a steady increase in the number of cells infected by N-MLV was seen with increasing amounts of APs. It was possible to raise the infectivity of the N-tropic virus to a level that was almost equal to the B-tropic virus, implying that addition of APs containing 800ng CA could approach saturation of Trim5α restriction (Figure 3.2a).

In contrast, it proved much more difficult to saturate $Fv1^n$ restriction in NIH 3T3 cells (Figure 3.2b). Addition of APs containing B-tropic CA clearly reduced restriction of B-MLV while having little effect on N-MLV. However, even with the highest concentration of added virus, an appreciable difference in titres of N-MLV and B-MLV was still apparent. This can probably be attributed to the relatively high levels of $Fv1^n$ present in NIH-3T3 cells (Yap and Stoye, 2003).

With Balb/3T3 cells, almost complete abrogation of $Fv1^b$ restriction was seen by N-tropic APs (Figure 3.2c). However, addition of N-tropic particles also increased the apparent titre of B-tropic MLV. This is consistent with the previous observation of low-level restriction of B-MLV by $Fv1^b$ when over expressed (Bock et al., 2000).

Given that for both TE671 and Balb/3T3 cells restriction is relatively easily saturated, knowing the quantity of virus added becomes very important. Abrogating particles must be added at sub-saturating conditions in order to detect small changes in their ability to interact with the restriction factor.

Therefore in the experiments described below to examine the ability of CA to interact with Fv1 and Trim5α, APs containing 800ng CA were added to TE671 and Balb/3T3 cells, and 1700ng to NIH T3 cells.

3.2 Gag processing and maturation requirements in the abrogation of Fv1 and Trim5α restriction

3.2.1 Gag processing and core assembly mutants

With a view to the examination of Gag processing/maturation requirements for the interaction of the virus with the restriction factor, a series of mutations, described in Table 3.1, were introduced by site directed mutagenesis into the *gag-pol* constructs used to produce N- and B-tropic APs. This series comprised active site mutations in PR and RT, changes inhibiting MA-p12, p12-CA and CA-NC processing as well as a mutation preventing the formation of a salt bridge between residues P1 and D54 of CA critical for the assembly of processed CA into mature cores (Rulli et al., 2006; von Schwedler et al., 1998).

The effect on processing of these mutations have previously been described in detail however, to confirm the patterns of protein expressions in virions prepared using these plasmids, Western blot analysis of released virus, normalised by ELISA and concentrated by ultracentrifugation was performed, using anti-CA and anti-p12 antibodies. Results with both N-tropic and B-tropic virions are shown, and are comparable. Generally results with both N-tropic and B-tropic virions were consistent with published data (Figures 3.3 and 3.4)(Oshima et al., 2004).

Thus, after probing with anti-CA a band was observed with the mobility of wild type CA in RT-, MAxp12, and D54A; a band of the size predicted for unprocessed Gag in PR- and bands corresponding to p12-CA and CA-NC in p12xCA and CAxNC. Somewhat surprisingly, there was a significant amount of apparently uncleaved Gag precursor in the RT- mutant (Figure 3.4a,b)

The results with anti-p12 (Figure 3.4c,d) were slightly less straightforward, and complicated in part by the incomplete processing of MA-p12 seen in wild type virus and the apparent non-reactivity of our anti-p12 sera with full length Gag. However Figure 3.4c,d does reveal bands of the expected size in MAxp12 (27kDa) and p12xCA (42kDa) and is consistent with reduced Gag processing in RT- (compare relative intensities of the 12 and 27kDa bands in wt and RT-). As expected, the D54A assembly mutant processed Gag normally (Figure 3.4e,f)

Name	Mutation	Predicted Phenotype	Ref.
RT-	D224E (in RT)	Inactive RT	(Lowe et al., 1991)
PR-	D32L (in PR)	Inactive PR	(Fu and Rein, 1993)
MAxp12	Y129D (in gag)	Inhibition of MA-p12 processing	(Oshima et al., 2004)
p12xCA	F214D (in gag)	Inhibition of p12-CA processing	(Oshima et al., 2004)
CAxNC	L477R (in gag)	Inhibition of CA-NC processing	(Oshima et al., 2004)
CA D54A	D268A (in gag)	No core formation	(von Schwedler et al., 1998)

Table 3.1 Mutation, Predicted Phenotype and Previous Characterisation of MLV Gag Mutants

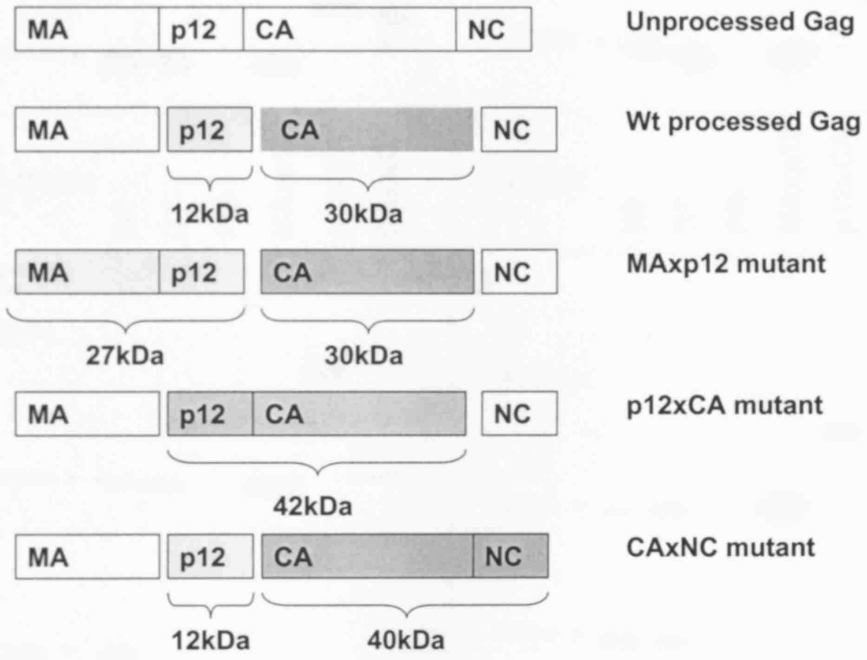
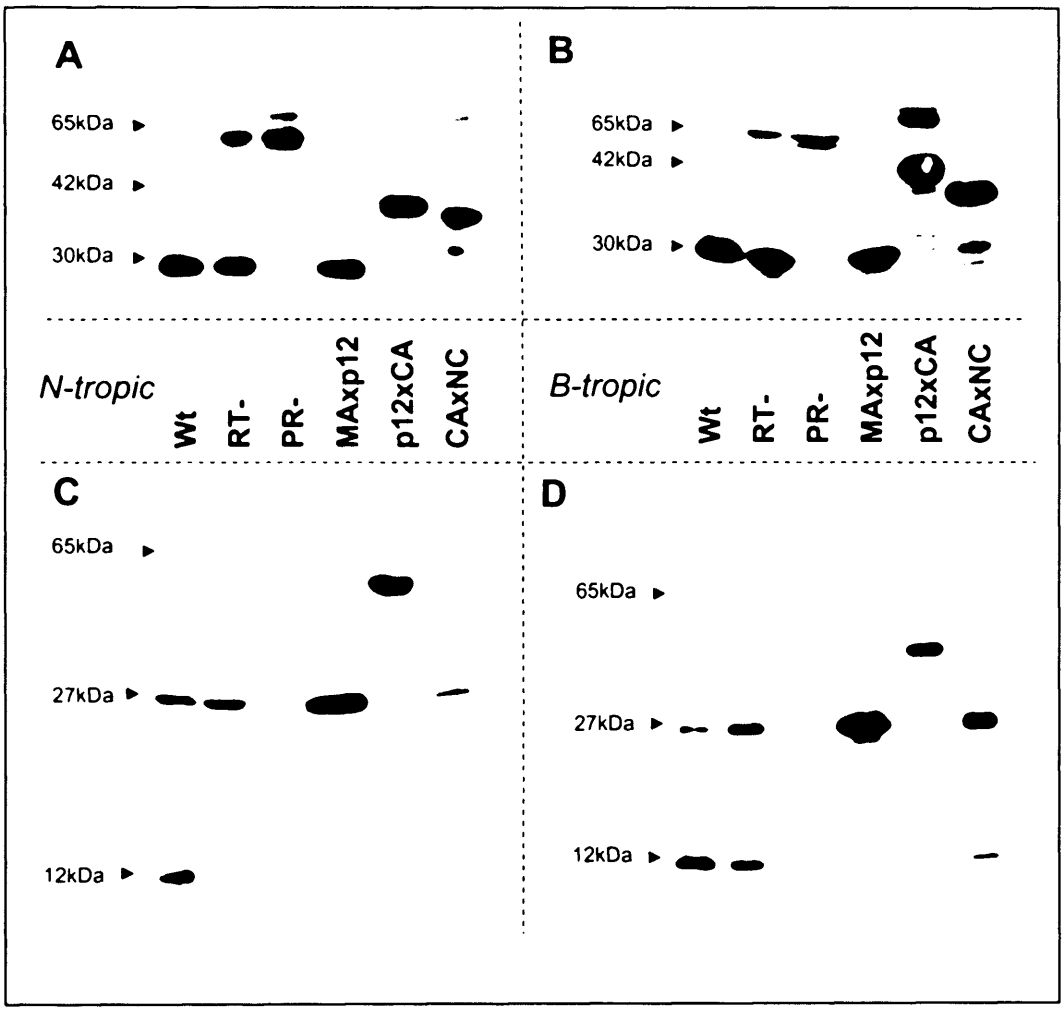


Figure 3.3 Predicted proteolytic processing in Gag mutants.
Sizes of predicted p12 and CA containing fragments are indicated for comparison to figure 3.4



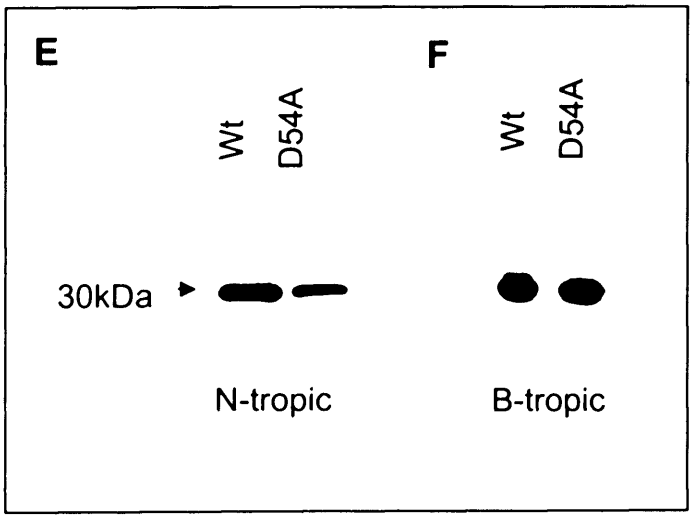


Figure 3.4 CA expression and processing in mutant viruses.
After normalisation by CA ELISA, virus containing supernatants were centrifuged to pellet the virions, re-suspended in SDS/loading dye and separated on an SDS/PAGE gel. After transfer to membranes, blots were probed with monoclonal anti-CA antibodies (A, B, E, F) or polyclonal anti-p12 sera (C, D). For clarity and ease of interpretation, molecular weights shown here show the sizes of the Gag processing products rather than the markers used.

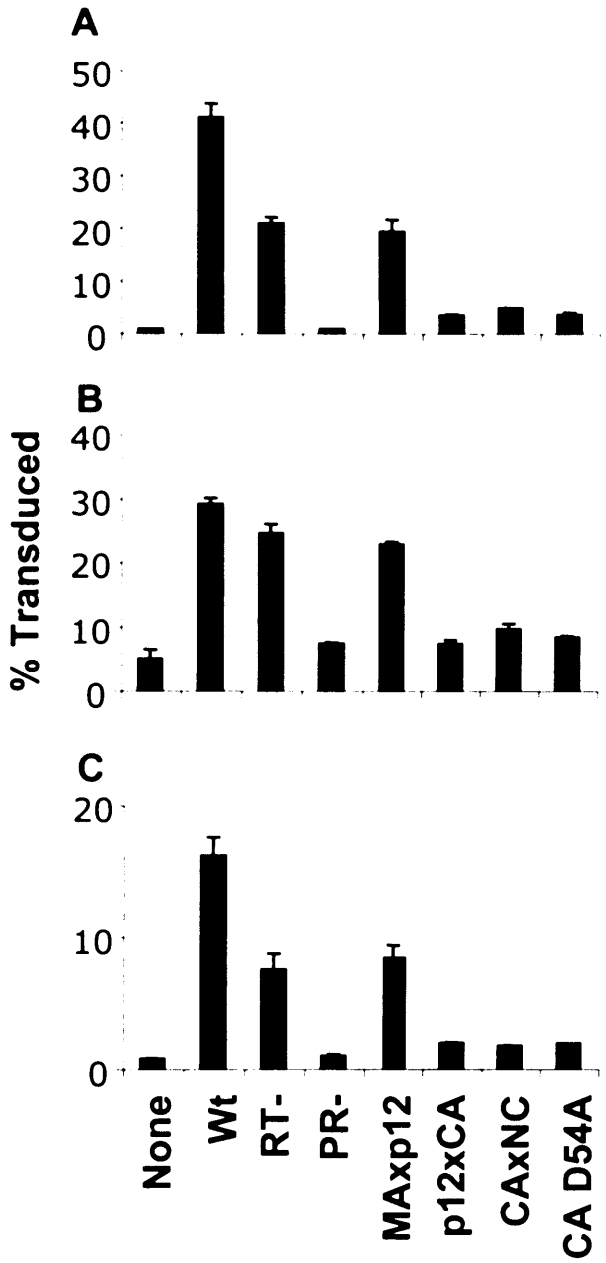


Figure 3.5. Abrogation properties of mutant viruses.
$4x10^4$ cells were pre-treated with 800ng N-tropic VLPs (TE671 (a) and Balb/3T3 (b)) or 1700ng B-tropic VLPs (NIH 3T3 (c)) prior to infection with a fixed amount of N-tropic (TE671 and Balb/3T3) or B-tropic (NIH 3T3) virus carrying the eGFP vector. The percentage of transduced cells was determined by flow cytometry. Data is the mean of 3 experiments. Error bars show standard deviation.

3.2.2 Abrogation properties of Gag mutants

The abrogation properties of the panel of viruses were examined. Cells were treated with APs containing restricted CA for 3 hrs and then infected with restricted virus. EGFP positive cells were counted three days later. Results from such an experiment are shown in Figure 3.5. With all three cell types significant abrogation was seen only with WT, RT- and MAxp12 CA. Abrogation was abolished, essentially completely, by the PR-, p12xCA, CAxNC and D54A mutations. These findings imply that for abrogation to occur, complete processing at both the N- and C-termini of CA is necessary. Further, the absence of abrogation with D54A hints at a possible role for assembly in addition to processing.

3.3 Discussion

The experiments described here characterise abrogation of 3 different restriction factors by both wild-type and mutant viruses. It was shown that the Trim5α restriction in TE671 cells can be almost completely saturated by addition of a sufficient amount of AP. A similar situation applies in the case of $Fv1^b$ in Balb 3T3 cells. Using equivalent amounts of virus however it was not possible to saturate restriction by $Fv1^n$ in NIH 3T3 cells. This is probably attributable to the relatively high level of expression when compared to $Fv1^b$, and may suggest that the saturability of a particular restriction factor is linked to its expression level. Consistent with this proposition, it is not possible to saturate restriction mediated by over-expressed Fv1 (M. Bock, unpublished data).

Mutations blocking MA-p12 processing or eliminating RT activity only had a very limited effect on the ability to abrogate restriction despite the fact that these mutations cause a significant loss of infectivity. The data from the RT mutant indicate that abrogation can still occur despite the inability of virus to produce an active reverse transcription complex. Fv1 is known to block the virus at a stage after reverse transcription. This may suggest that Fv1 interacts with the virus at a stage prior to reverse transcription but only has a phenotypic consequence at a post RT stage.

It was demonstrated that for a virus to effectively abrogate restriction, the CA protein must be separated from both the p12 and NC components of Gag, a process mediated by the viral protease. Mutations either preventing proteolytic processing completely or specifically blocking processing at these points resulted in the production of virions incapable of abrogation. One possibility is that a structural rearrangement occurs within CA upon processing which allows it to interact with the restriction factors, indeed proteolytic processing does cause the refolding of the amino terminus and the formation of the P1-D54 salt bridge.

An alternative explanation however stems from the observation that p12xCA and CAxNC virions have been shown to have a defect in core formation; they do not form a condensed CA core visible by transmission electron microscopy (Oshima et al., 2004). Figure 3.6 shows a figure extracted from the paper by Oshima and colleagues documenting these blocks. These data raise the possibility that core formation may be required for CA interaction with restriction factor. The lack of abrogation seen with the D54A core assembly mutant is consistent with this hypothesis.

The next chapter describes experiments designed to address this possibility that core assembly is required for restriction factor/virus interaction.

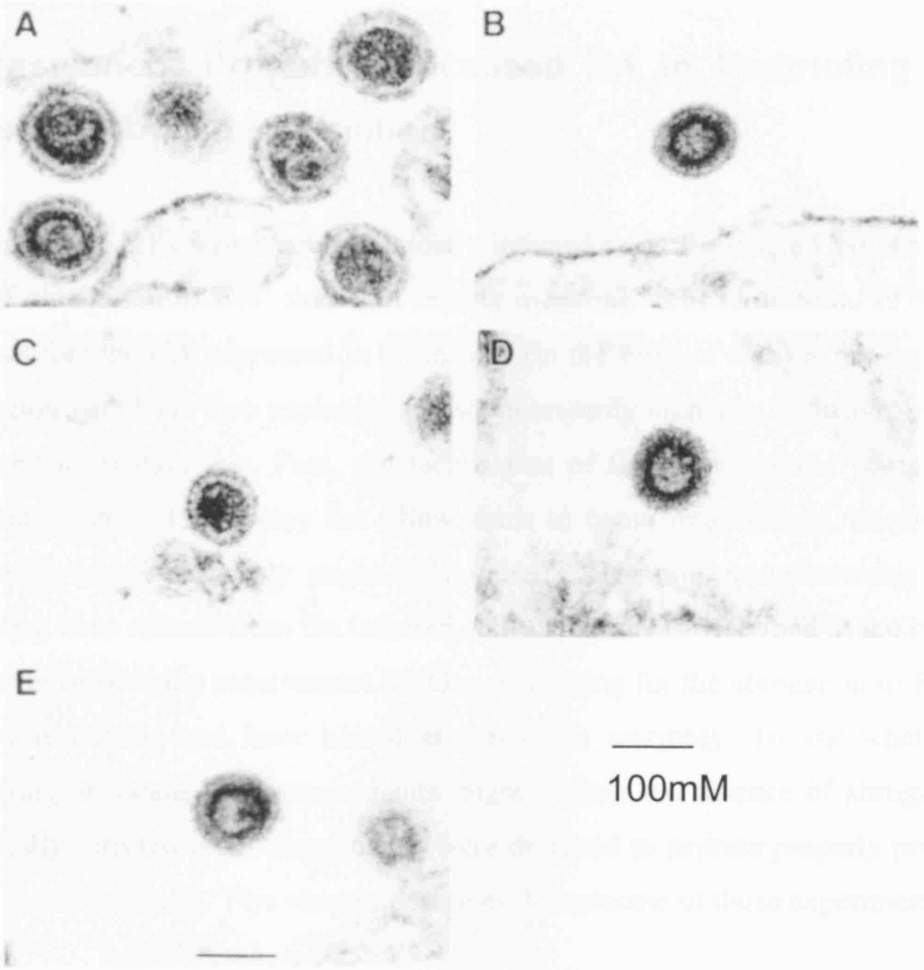


Figure 3.6 Thin-section electron micrographs showing morphology of Gag processing mutants.
(a) Wt (b) PR- (c) MAxp12 (d) p12xCA (e) CAxNC
Figure is taken directly from Oshima *et al.* (2005).

CHAPTER 4

Expression of Properly Processed CA in Restricting Cells Does Not Abolish Restriction

Paradoxically, cells which are chronically infected with a restricted virus show no sign of abrogation of Fv1; they still restrict incoming virus (Jolicoeur, 1979). This suggests that the CA expressed in these cells (in the form of Gag) is not capable of interaction with Fv1. Two explanations, not necessarily mutually exclusive, might be advanced to explain this. First, the localisation of Gag in a cellular compartment different to that of Fv1 may not allow them to come into contact. Second, Gag processing and/or assembly might be required. Gag processing/assembly occurs following virus release from the infected cell. Experiments described in the previous chapter have shown a requirement for Gag processing for the abrogation of Fv1 and Trim5α restriction and have hinted at a role for assembly. To ask whether the processing or localisation requirements might explain the absence of abrogation in chronically infected cells, experiments were designed to express properly processed CA in restricting cells. This chapter describes the outcome of those experiments.

4.1 Use of the Foot and Mouth Disease Virus 2A peptide to produce properly processed CA with an N-terminal proline

Upon Gag processing by the virus protease, the CA protein released has a proline residue upon its N-terminus. In the mature form of CA, this proline residue forms a salt bridge with an aspartate residue at position 54 of CA stabilising a β-hairpin structure at the N-terminus of CA. The formation of this salt bridge has been shown to be essential for proper core formation (von Schwedler et al., 1998). In addition, data in the previous chapter has also suggested that the formation of this salt bridge is essential for the virus to interact with the restriction factor. Given the potential importance of the N terminal proline (in core formation or otherwise), any attempt to express processed CA in cells must produce the protein with a N terminal proline

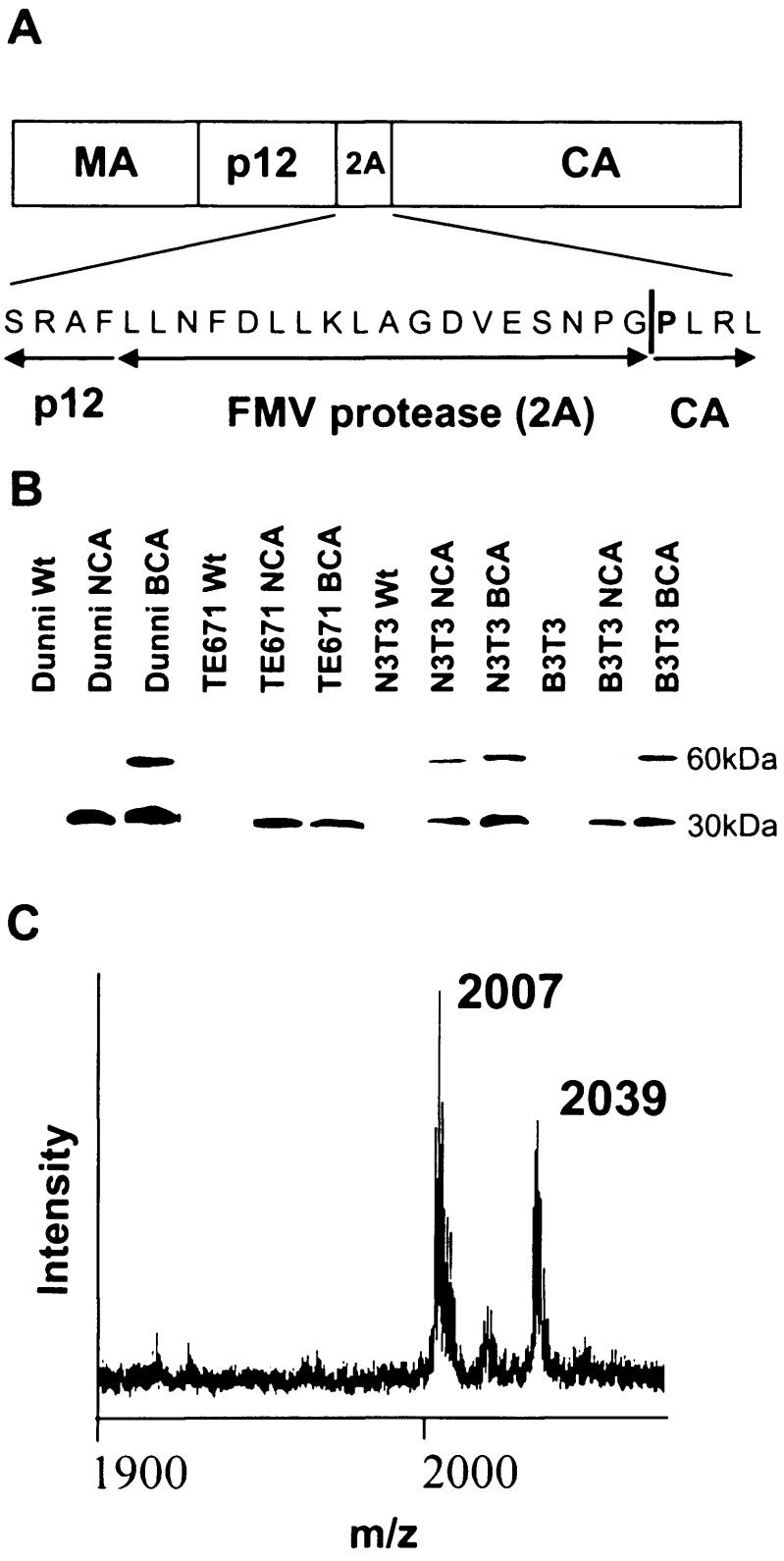


Figure 4.1 FMDV 2A processed CA.
(a) Diagram showing sequence, position and cleavage point of FMDV 2A protease. Cut is between final Gly of the protease and the first Pro of CA. (b) Western blot analysis using anti-CA antibodies of cell lysates from wild type, NCA and BCA expressing clones. The band at 30kDa is due to processed CA, the band at 60kDa is the unprocessed precursor. (c) Mass spectrum showing fragments detected from Asp-N digestion of immunoprecipitated CA from dunni NCA cells.

rather than methionine. In order to achieve this aim the Foot and Mouth Disease (FMDV) 2A sequence was employed.

The FMDV 2A sequence was originally thought to be a protease. However it is now believed to mediate a co-translational event described as a 'ribosomal skip' between the VP1-2A and 2BC domains of a poly-protein produced by the virus, preventing formation of a Gly-Pro peptide bond (Donnelly et al., 2001). Work by Ryan has shown that a 19 amino acid oligopeptide found at the C-terminus of the 2A protein is sufficient to allow this processing even in the context of another pair of proteins (Ryan and Drew, 1994; Ryan et al., 1991). Conveniently, the last amino acid in this sequence is a proline. The processing event occurs between this proline and a glycine on its N-terminal side. The resultant C terminal product released therefore has a proline on its N-terminus. The 2A oligopeptide was introduced between p12 and CA in a construct consisting of MA, p12, 2A and either N or B tropic CA and subcloned into a retroviral expression vector based on pLIB (Clontech) by M. Bock (pLIB-NCA-puro, pLIB-BCA-puro) (Figure 4.1a). When expressed, the predicted product is properly processed CA with an N-terminal proline available for salt bridge formation.

4.2 Expression and characterisation by mass spectrometry of CA produced by pLIB-NCA-puro and pLIB-BCA-puro

In order to examine expression of the NCA/BCA constructs, TE671, Balb/3T3, NIH 3T3 and *M.dunni* cells were transduced with the retroviral vectors expressing each of these constructs. Single-cell clones were derived as described in *Materials and Methods*.

Cell lysates from these clones were subjected to analysis by western blot probing with a monoclonal antibody directed against CA. In all of the cell extracts, the expected band at 30kDa could be observed. In several of the lines, unprocessed 60kDa material was also observed presumably corresponding to unprocessed p15-p12-2A-CA.

Given the potential importance of the N-terminal proline for the formation of a salt bridge with D54 and the requirement of this salt bridge for abrogation, it was important to ensure that this proline was still present after processing. CA from an

M.dunni NCA-expressing clone was immunoprecipitated, digested with Asp-N and analysed by mass spectrometry. Asp-N cleaves at the N-terminal side of Asp residues and would be predicted to release a fragment with mass/charge (m/z) ratio of 2007 derived from the N-terminus of CA made up of PLRLGGNGQLQYWPFSSS. This fragment was observed. Commonly, tryptophan residues in these fragments are oxidised and therefore peaks appear as doublets, containing a second peak with an m/z ratio greater by 32. This was also observed at 2039. The 2A protease therefore releases CA processed precisely as predicted. There was no evidence of alternate processing.

4.3 Immunofluorescence analysis of processed N and B CA

In order to confirm that CA expressed by these constructs is able to move freely throughout the cell and potentially interact with any restriction factor, the localisation of CA in these cells was determined by immunofluoresence and compared to that observed in the context of Gag in chronically infected cells.

Cells were examined by immunostaining for CA (Figure 4.2b-i). In all of the cell lines, diffuse CA staining was observed, with some localisation in punctate structures. Nuclear staining was also observed. A 30kDa protein could potentially diffuse freely into the nucleus through the nuclear pores. This is distinct from the staining observed in chronically infected cells where CA is confined to perinuclear and plasma membrane compartments (Figure 4.2a).

The cell lines were also examined by immunostaining with a monoclonal antibody directed against p12 to detect processed MA-p12-2A and any unprocessed MA-p12-2A-CA (Figure 4.3b-i). p12 localisation in the cells was comparable to that observed for Gag in chronically infected cells (Figure 4.3a).

Taken together, these data suggest that upon processing, CA released from the NCA/BCA constructs is freed from the perinuclear and membrane localisations imposed by the presence of MA and thus able to move freely in the cytoplasm where it may potentially interact with any restriction factor.

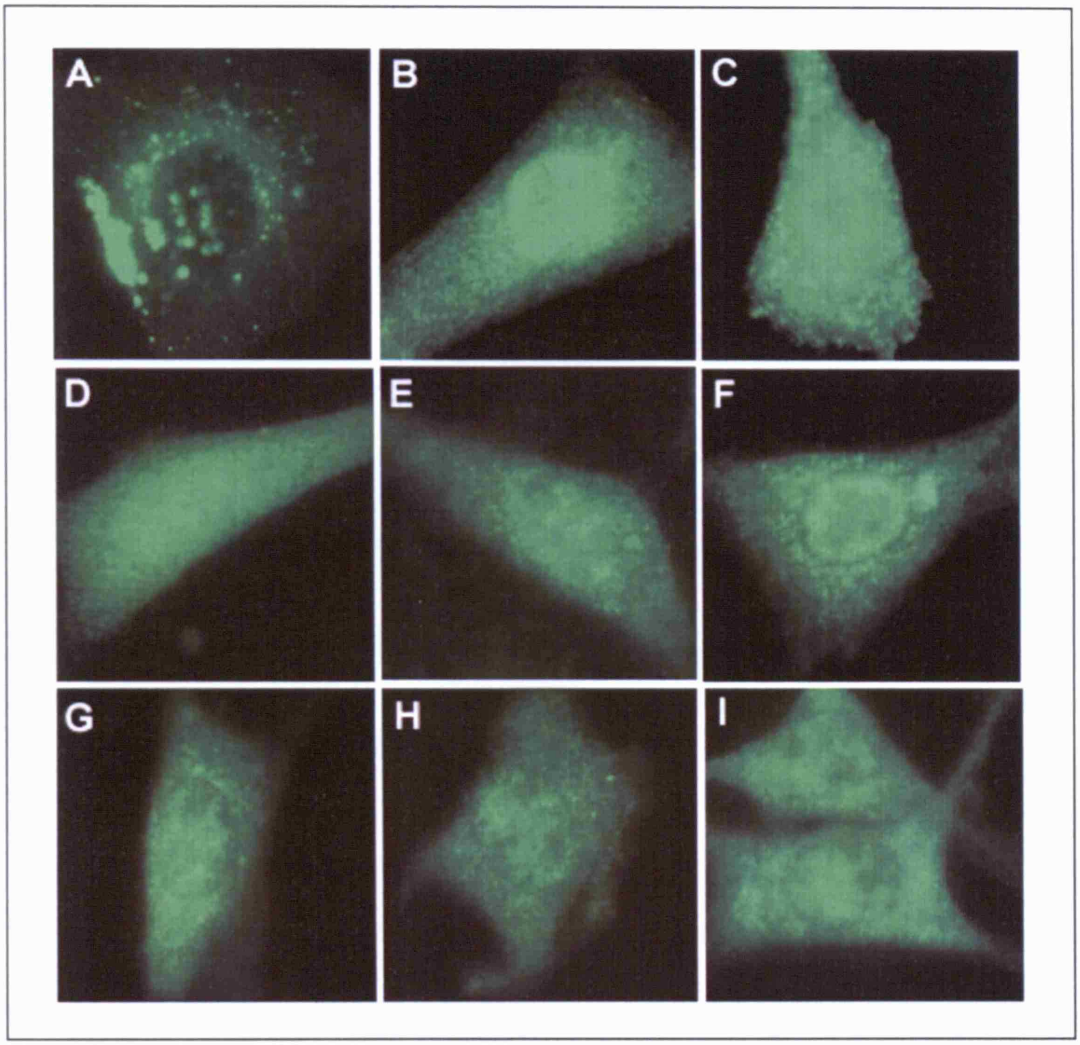


Figure 4.2 Immunostaining for CA
(a) *M.dunni* cell chronically infected with N-MLV, (b) *M.dunni* cell expressing NCA, (c) *M.dunni* cell expressing BCA, (d) TE671 cell expressing NCA, (e) TE671 cell expressing BCA, (f) NIH 3T3 cell expressing NCA, (g) NIH 3T3 cell expressing BCA, (h) Balb/3T3 cell expressing NCA, (i) Balb/3T3 cell expressing BCA.

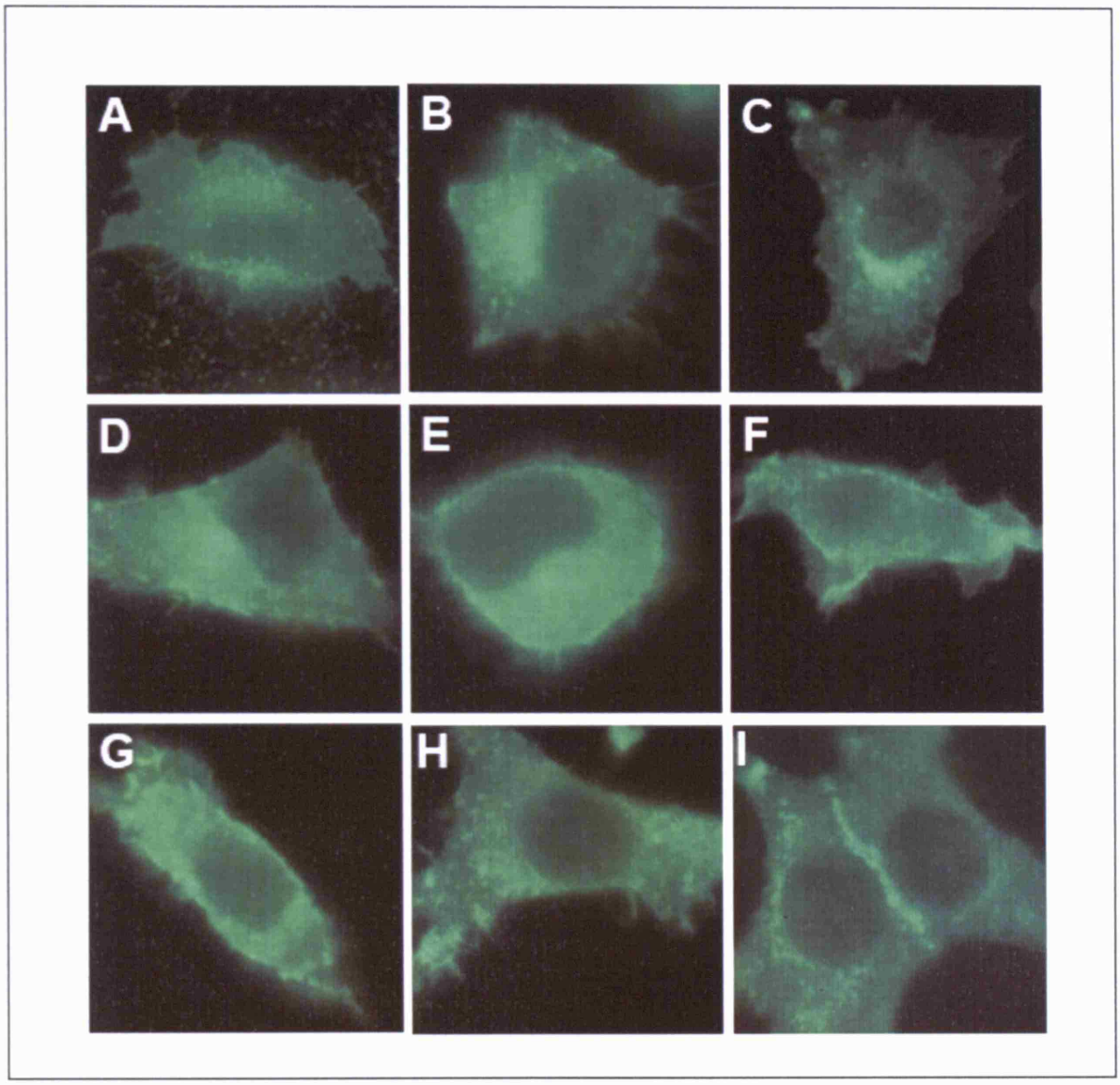


Figure 4.3 Immunostaining for p12
(a) *M.dunni* cell chronically infected with N-MLV, (b) *M.dunni* cell expressing NCA, (c) *M.dunni* cell expressing BCA, (d) TE671 cell expressing NCA, (e) TE671 cell expressing BCA, (f) NIH 3T3 cell expressing NCA, (g) NIH 3T3 cell expressing BCA, (h) Balb/3T3 cell expressing NCA, (i) Balb/3T3 cell expressing BCA.

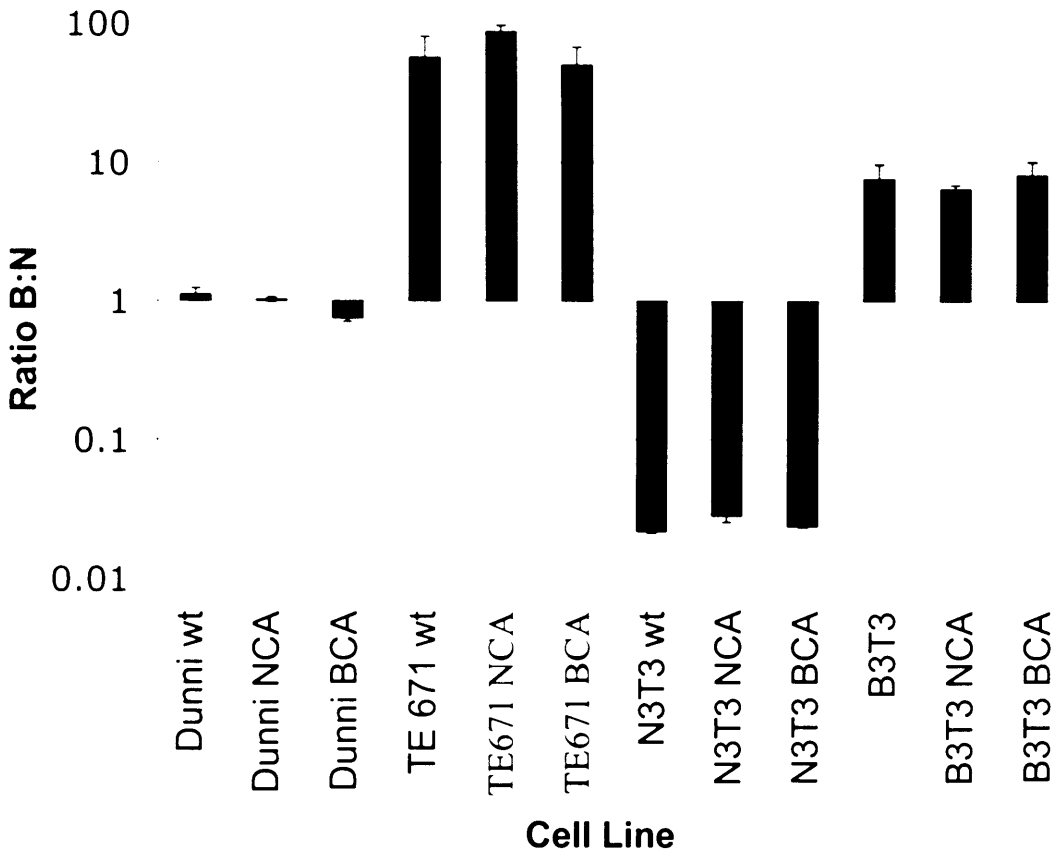


Figure 4.4 Restriction characteristics of cell lines expressing processed N-tropic or B-tropic capsid.
Cells were challenged with equal amounts of both N and B MLV carrying a GFP vector and the ratio of percentage transduced cells (B:N) was determined.

4.4 Expression of restricted properly processed CA does not abrogate restriction of incoming virus

To determine the ability of CA-expressing cells to restrict MLV infection, these cells were challenged with equal amount of N- and B-tropic virus carrying the pLNCG (eGFP) vector. The ratio of the percentage transduced with N and B tropic virus was determined. A non-restricting cell line would be transduced equally well by both the N and B tropic viruses and so would give a ratio of one. Cell lines restricting the N tropic virus would give a ratio >1 whereas cell lines restricting the B tropic virus would give a ratio of <1.

Titres of infecting viruses were equal on *M.dunni* cells that do not express an MLV restriction factor to giving a ratio of 1. This ratio was not affected by the expression of either N or B CA in these cells (Figure 4.4).

For the cell lines known to restrict the N-tropic virus (Balb/3T3 and TE671), wild type cells gave a ratio >1. This was unaffected by the expression of N or B CA in these lines. The converse applied to NIH 3T3 cells that restrict B-tropic MLV; wild type cells gave the expected ratio of <1 with the presence of N or B CA having no effect. Identical results were obtained using a second set of independently derived clones. These data indicate that intracellular expression of fully processed CA is insufficient to abrogate restriction.

4.5 Discussion

The data described show that cells lines transduced with the pLIB-NCA/BCA-puro constructs produce properly processed MLV CA of the correct size and N-terminal sequence. It appears that this CA when processed is free to move throughout the cytoplasm of the cell and also into the nucleus. It is no longer constrained by the localisation of Gag.

The localisation of *functional* Fv1 and Trim5α not completely clear. Evidence suggests that both are cytoplasmic proteins. Fv1 has been observed in association with the trans-golgi network whilst Trim5α is found in puncta within the cytoplasm often described as cytoplasmic bodies. It is likely therefore that the free processed CA should be capable of coming into contact with both restriction factors. However

it should be noted that the possibility that Fv1 and Trim5α are compartmentalised in such a way as to exclude even free CA cannot be formally excluded.

Experiments described in Chapter 3 suggested that in order for an incoming virus to interact with and abrogate either Trim5α or Fv1 restriction, it is necessary for CA to be properly processed and/or assembled. This was also offered as a possible explanation as to why chronically infected cells still restrict incoming virus. The data here show that processing alone is not sufficient. Expression of processed, non-assembled CA in restricting cells had no effect on their ability to restrict incoming virus. This suggests that CA must also be processed and importantly *assembled* into a polymeric core before interaction with either Fv1 or Trim5α can occur.

CHAPTER 5

Polymeric CA material in Restriction Factor Binding and Stability assays

Data from the previous two chapters strongly suggest a requirement for CA to be in its mature polymeric form, composing a virus core, in order to be able to interact with restriction factors. This offers a compelling explanation as to why previous attempts to show interaction using CA in its monomeric form were not effective.

It has been possible to isolate internal retroviral material by removing the lipid membrane, for many years. Treatment of RSV with Triton-X, followed by centrifugation released ribonucleoprotein complexes from virions (although not intact cores) (Coffin and Temin, 1971) which sedimented to a density in sucrose/D_20 of 1.34g/ml. Later, it was shown to be possible to isolate complexes closely resembling those found within the virion of ALV and Friend MLV (Bolognesi et al., 1973). This early work by Bolognesi and colleagues isolated ALV and Friend MLV core material by centrigfugation through a layer of detergent (Sterox-SL) atop a sucrose gradient, with core matieral found at an equilibrium density of 1.2-1.26g/ml sucrose. Around the same time, work by Schafer and colleagues showed the Friend MLV could also be isolated by incubating virions with ether prior to centrifugation (Lange et al., 1973). Termaoto used similar procedures to isolate MMTV cores, concluding that they could obtain maximal yields using 0.5%-1% Triton-X to remove the lipid membrane (Teramoto et al., 1977). Somewhat more recently is was shown to be possible to isolate intact cores from EIAV and HIV-2 (Kewalramani and Emerman, 1996; Roberts and Oroszlan, 1989). HIV-1 cores were first isolated by Welker and colleagues by incubating virions in Triton-X and either capturing the liberated complexes in sucrose gradients or simply pelleting in a centrifuge (Forshey and Aiken, 2003; Welker et al., 2000). It was however necessary to perform this procedure very quickly to avoid disruption of the core. Forshey and Aiken later used isolated HIV-1 cores to perform *in vitro* disassembly reactions.

The work described above indiates that retroviral cores can be successfully isolated from virions. This efficiency of this process however is dependent upon the precise details of the extraction prodcedure and the detergent used. Despite this, isolation of

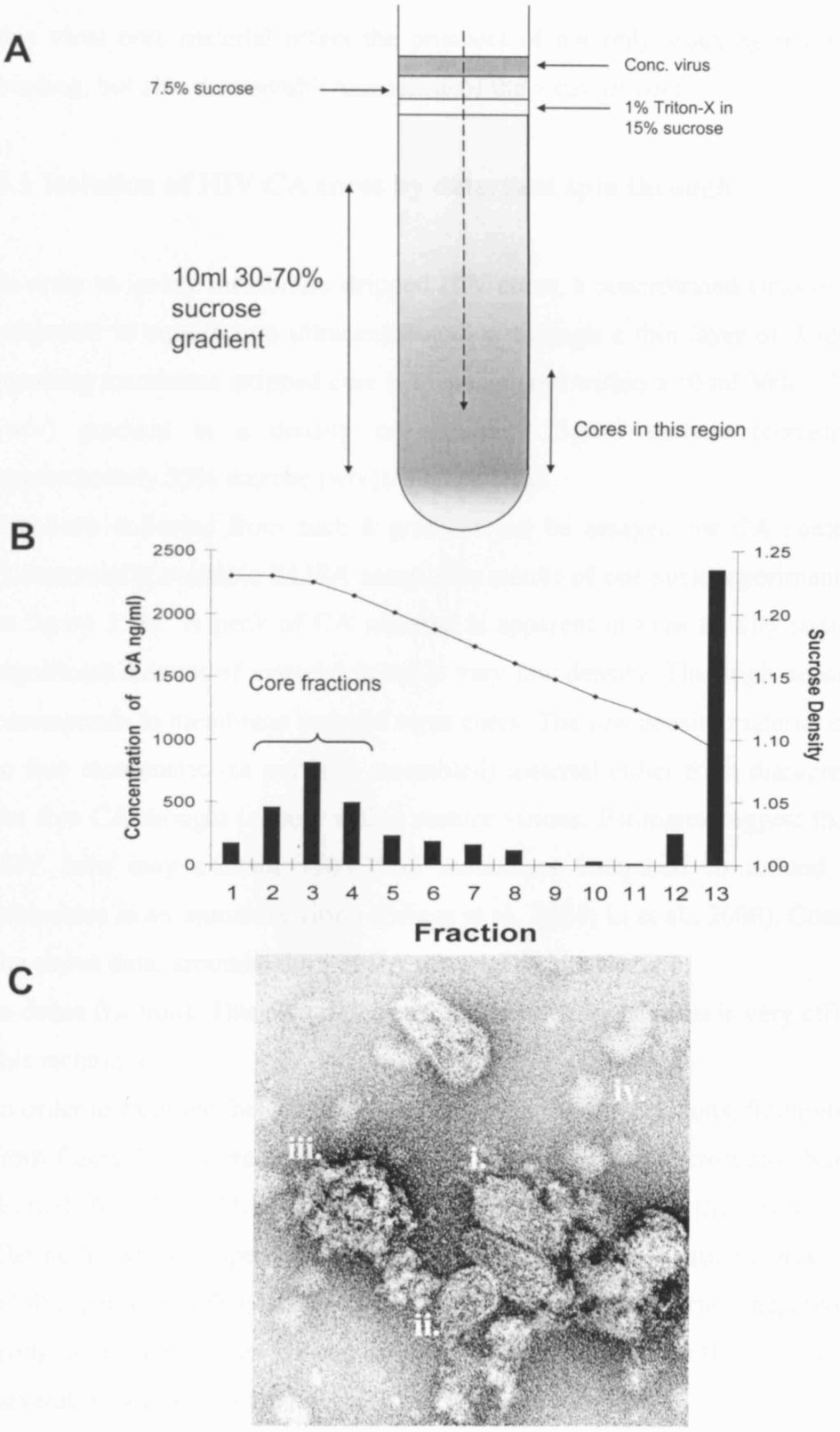


Figure 5.1 Preparation of HIV capsid cores.
(a) Schematic of gradient used to prepare cores. (b) Graph showing CA concentration (as determined by ant-HIV CA ELISA) and sucrose density of 1ml fractions collected from an HIV-1 gradient. (c) Negative stain electron micrograph showing fixed pelleted material from fractions 2, 3 and 4 of the above gradient.

this virus core material offers the prospect of not only studying restriction factor binding, but also disassembly/uncoating of the virus, *in vitro*.

5.1 Isolation of HIV CA cores by detergent spin through

In order to isolate membrane stripped HIV cores, a concentrated virus preparation is subjected to equilibrium ultracentrifugation through a thin layer of detergent. The resulting membrane stripped core is then captured within a 10 ml 30% - 70% sucrose (w/v) gradient at a density of around 1.23g/ml sucrose (corresponding to approximately 55% sucrose (w/v)) (Figure 5.1a).

Fractions collected from such a gradient can be assayed for CA content using a commercially available ELISA assay. The results of one such experiment are shown in figure 5.1b. A peak of CA material is apparent in high density sucrose, with a significant amount of material found at very low density. This high density material corresponds to membrane stripped virus cores. The low density material corresponds to free monomeric (or partially assembled) material either from disrupted cores, or the free CA thought to exist within mature virions. Estimates suggest that a mature HIV core may contain 1500-2000 monomers compared to around 5000 Gag molecules in an immature virion (Briggs et al., 2004; Li et al., 2000). Consistent with the above data, around a third of CA material can be isolated
in dense fractions. This also suggests that the recovery of cores is very efficient using this technique.

In order to examine the composition of the high density fractions, fractions 2, 3 and 4 from figure 5.1,b were pooled and examined by electron microscopy. Samples were diluted 10 fold in STE buffer then centrifuged again to pellet the assembled material. The pellet was resuspended in 100µl of STE, then fixed by adding an equal volume of 4% paraformaldehyde. Samples were placed on EM grids, negatively stained using uranyl acetate and viewed using an electron microscope (Figure 5.1, c).

Several types of material were observed. An example of each is noted on the micrograph. A significant number of apparently intact conical cores were clearly present (i), as well as partially disrupted cores (ii). Whether these cores were disrupted by detergent stripping or preparation for EM is not clear. Some amorphous material of unknown origin was also present (iii). Some residual sucrose was also

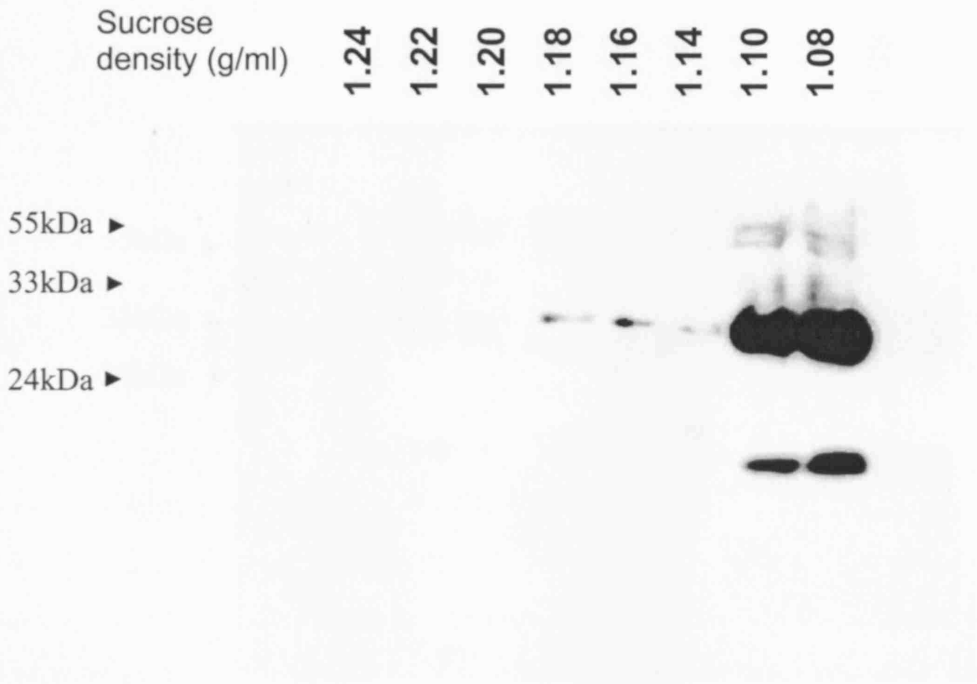


Figure 5.2 An attempted MLV core preparation.
Concentrated 293T cell supernatant containing N-MLV was spun through detergent as described in figure 5.1. 1.5ml fractions were collected from the gradient and analysed by western blot for CA using a polyclonal antibody directed against MLV CA . Sucrose density was determined from the refractive index of the fraction.

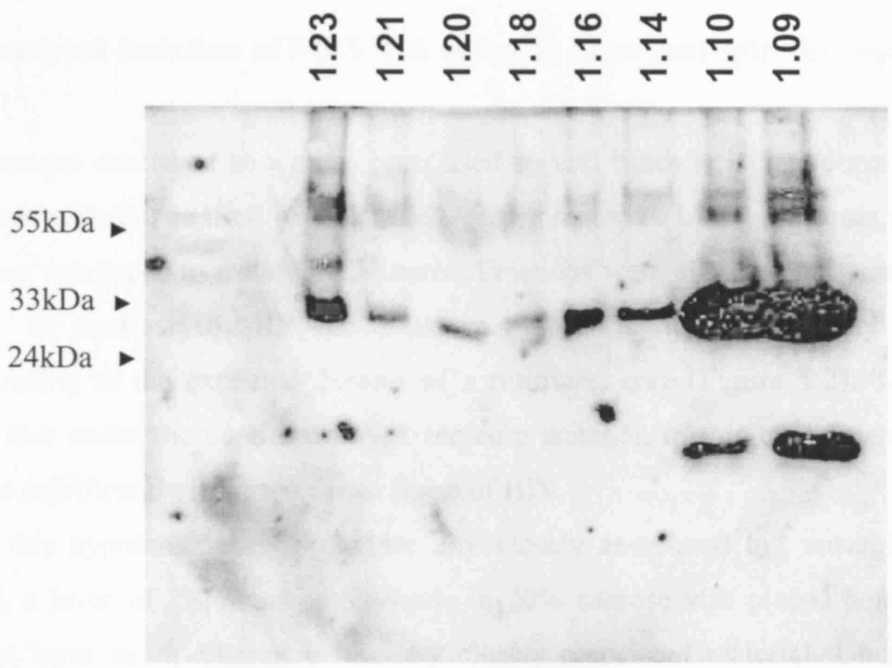


Figure 5.3 Isolation of MLV Core Material By Spin Through 2% Paraformaldehyde
Concentrated 293T cells supernatant containing N-MLV was spun through detergent as described in figure 5.1. Below the layer of detergent was placed a layer of 2% paraformaldehyde in 20% sucrose 1.5ml fractions were collected from the gradient and analysed by western blot for CA using a polyclonal antibody directed against MLV CA. Sucrose density was determined from the refractive index of the fraction.

observed as globular accumulations (iv). Whilst the precise composition of the high density peak fractions is not completely defined, CA is clearly present (as shown by ELISA), is assembled into a higher order structures (shown by the manner of its preparation) and can readily be observed in the form of cores by electron microscopy.

5.2 Attempted isolation of MLV CA cores by detergent spin through

The technique described above has been used several times to isolate cores but has never been reported as used to isolate MLV core material. Using the same protocol an attempt was made to isolate MLV cores. Fractions were analysed by western blot for CA. In contrast to HIV, no peak was observed in high density sucrose corresponding to the expected density of a retroviral core (Figure 5.2). This may suggest that under the conditions used for core isolation in this experiment, MLV cores are significantly less stable than those of HIV.

To test this hypothesis and to capture any closely associated but unstable MLV material, a layer of 2% paraformaldehyde in 20% sucrose was placed beneath the detergent layer in an attempt to fix any closely associated material. Under these circumstances, it was possible to isolate a small proportion of material in high density sucrose consisting of some unprocessed Gag and processed 30kDa CA (Figure 5.3). When examined by EM however no structure of any defined shape was observed suggesting that any material isolated is already in a partially disassembled state (data not shown). This data suggests that, at least in the conditions used in this preparation, MLV cores are less stable than those of HIV-1. Therefore it may be that the apparent faster uncoating observed for HIV-1 during an infection is not due simply to a difference in relative stability (Fassati and Goff, 1999; Fassati and Goff, 2001).

5.3 Reaction tubes and tips must be blocked with BSA to avoid loss of HIV CA material

During the course of these studies, it became apparent that after isolation of core material in sucrose, further manipulations resulted in a rapid loss of protein as

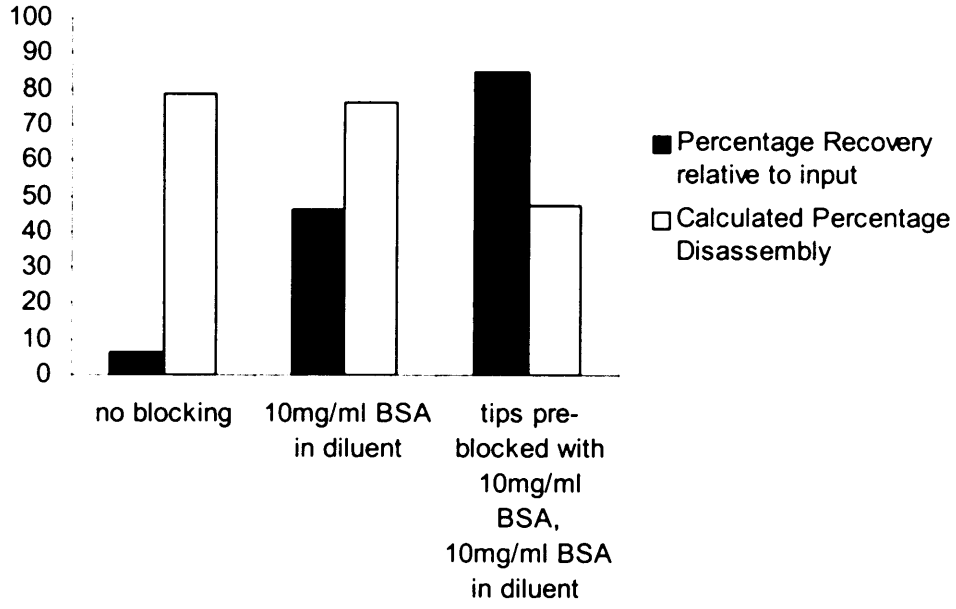


Figure 5.4 Effect of Blocking with BSA upon recovery of HIV CA material and Calculated Percentage Disassembly

15µl (22.5ng as determined by p24 ELISA) of HIV core material in sucrose was diluted with 285µl of STE, or STE 10mg/BSA at 4°C. One sample was handled with pipette tips pre-blocked with 10mg/ml BSA. Immediately after dilution, samples were subjected to ultracentifugation at 100,000g for 30 minutes to pellet core material. Samples were then re-suspended in 300µl STE or STE 10mg/ml BSA. Concentration of p24 was determined by ELISA and percentage recovery compared to input was calculated. Disassembly was calculated as amount in supernatant /(amount in pellet + amount in supernatant).

measured by ELISA. Given the very small quantities of material involved it seemed possible that some could be lost by binding to pipette tips and reaction vessels. To test this hypothesis, 22.5ng of isolated cores in sucrose were diluted with either STE buffer alone, or STE buffer containing 10mg/ml BSA. One BSA containing sample was also handled with pipette tips which had been pre-blocked with BSA and allowed to dry.

Immediately after dilution, samples were subjected to centrifugation at 100,000g to separate core material from free CA (as was planned for later assays). The supernatant was removed and the pellet resuspended in STE or STE with 10mg/ml BSA. The amount of CA in pellet and supernatant was determined by CA ELISA and compared to the input (Figure 5.4). With no BSA present, only around 7% of the input material was recovered. When BSA was included in the diluent, this proportion rose to 47%. When the sample was also handled with blocked tips, this proportion was further increased to 85%. In later experiments the presence of cell extracts rather than BSA allowed this proportion to rise to more than 95%.

Based on the ratio of material in pellet to supernatant, percentage disassembly can be calculated as amount in supernatant/(amount in pellet + amount in supernatant). Using these data, blocking with BSA decreased the apparent percentage disassembly from 80% to around 47%. In later experiments performed in cell extracts (with the highest recovery), disassembly was measured as less than 20% (see figure 5.9, time 0 mins) This loss of material appears to affect the pelleted CA more profoundly perhaps because pelleted material may be more affected by binding to plastic vessels due to concentration at the interface between the sample and the vessel. In addition, resuspension of the pelleted material requires extensive pipetting providing further opportunity for loss.

These experiments make it clear that recovery of input protein is of major concern when cell extract is not present. Recovery should be monitored closely and blocking agents used where possible.

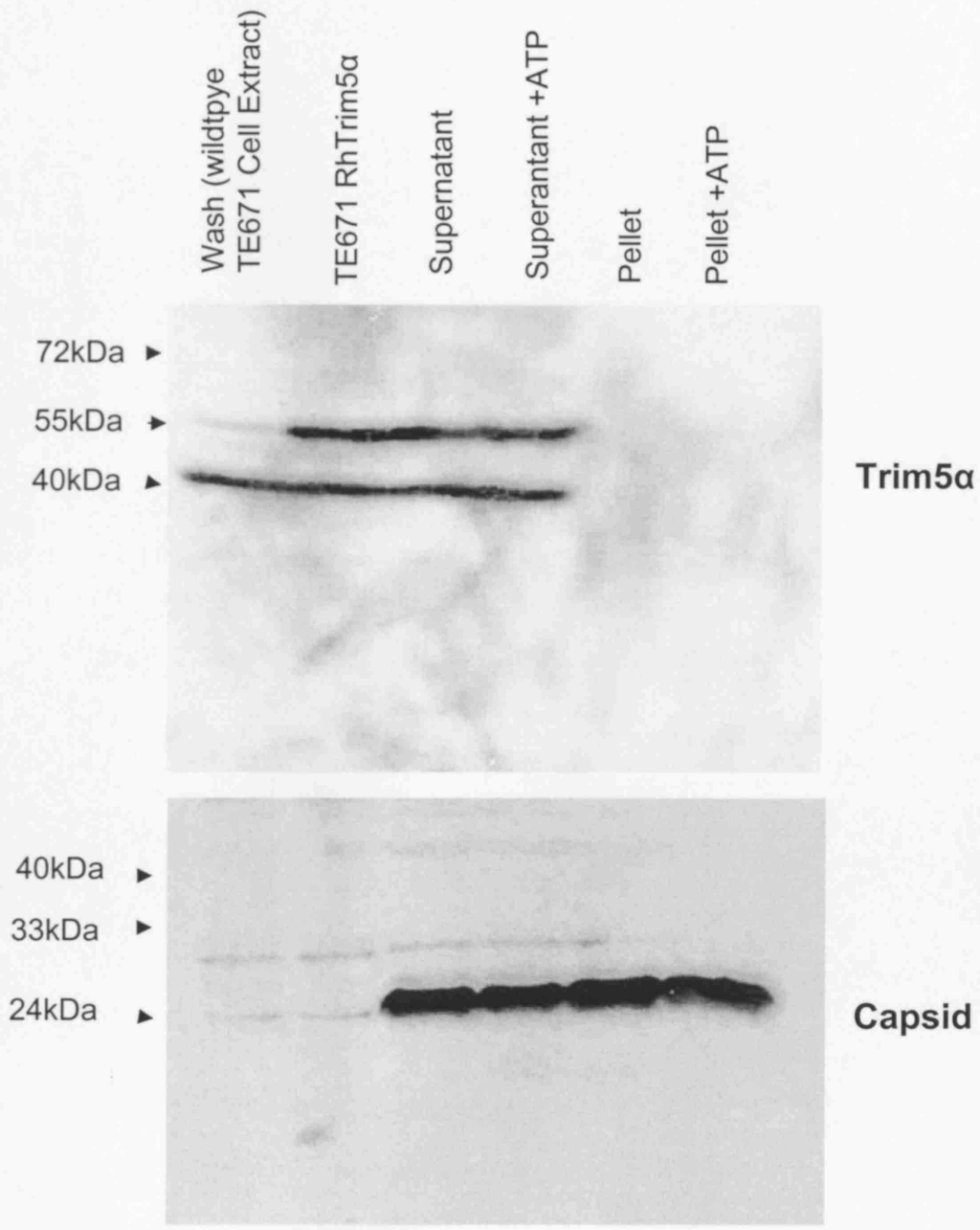


Figure 5.5 Attempted Pull Down of Trim5α using HIV core material
HIV core material was incubated with cell extracts containing expressing RhTrim5α with or without a supplement of 5mM ATP. After centrifugation at 16,000g or 30 minutee, the supernatants were removed and the pellets washed once with cell extracts not overexpressing Trim5α. Pellet and supernatant were analysed by SDS-PAGE followed by western blot for HIV CA and Trim5α.

5.4 Pull down assay for Trim5α using HIV core material

Experiments described in the previous chapters suggested that Trim5α may only interact with assembled, polymeric CA. In order to test this hypothesis, membrane stripped HIV core material was used in a pull down assay. In addition, others had speculated that ATP may be required for this binding (Gurer et al., 2005)(WI Sundquist, CSHL Retrovirus Meeting 2005).

A concentrated cell extract from TE671 cells stably expressing Trim5α from rhesus macaque was produced by two freeze-thaw cycles in STE buffer. Debris was cleared by centrifugation. The total protein content was determined by Bradford assay, and adjusted to 2.5mg/ml with STE buffer. One sample was supplemented with ATP to a final concentration of 5mM.

100µl core material (approximately 100ng of CA) was incubated with 200µl of cell extract at 4°C for 1 hour prior to centrifugation at 16,000g. The supernatant was removed and the pellet washed with a cell extract derived from wildtype TE671 cells to ensure that any co-factor necessary for binding would still be present in the wash (Endogenous, non restricting human Trim5α is undetectable in this extract, the band at around 55kDa shown is not derived from Trim5α. In addition, the lower band observed at around 38kDa does not appear to be due to Trim5 – see Chapter 7 for full details). The wash was removed and both the pellet and original supernatant were analysed by western blot for Trim5α and HIV CA.

Using this system, despite a significant amount of HIV CA being found in the pellet, no Trim5α was detected in either the presence or absence of ATP (Figure 5.5).

5.5 In vitro disassembly of HIV CA in cell extracts

Several hypotheses have been advanced as potentially describing the mechanism of action of Trim5α, the most prominent being that the binding of Trim5α causes premature or accelerated disassembly of the incoming capsid core. If this were the case, then the assay described above may not detect Trim5α binding, as binding would lead to disassembly of CA and consequently Trim5α would not be pulled down. In order to test this hypothesis, its was necessary to establish an assay in which the rate of disassembly of HIV CA cores in cell extracts could be determined.

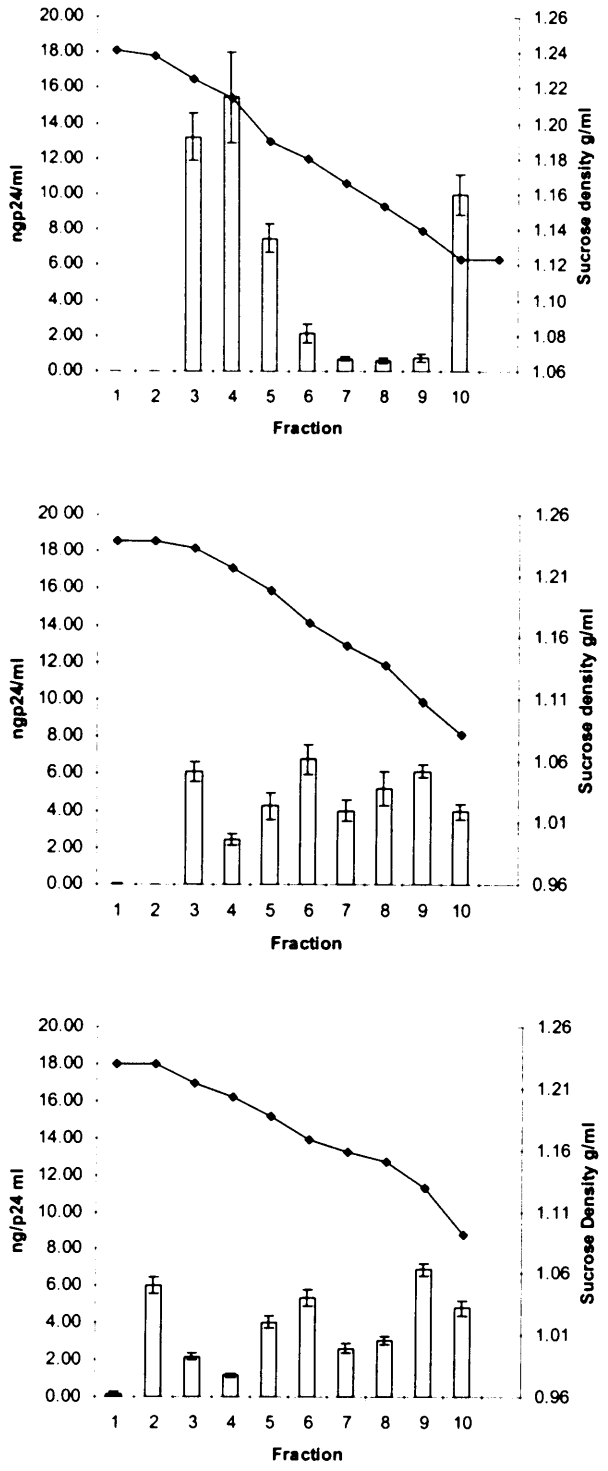


Figure 5. 6 Disassembly of HIV core material in cell extracts. HIV core material was incubated with cell extract from TE671 cells for 3 hours either at 4°C (top) or 37°C (middle), or TE671 cell extract containing Trim5α incubated at 37°C (bottom).The resulting mixtures were subjected to centrifugation through a 70%-30% sucrose gradient in a similar manner to that described in figure 5.1 (excluding the detergent layer). After 16 hours of centrifugation at 100,000g, fractions were collected and analysed by p24 ELISA. Sucrose concentration was determined from refractive index. Columns show mean and error bars show +/- standard deviation.

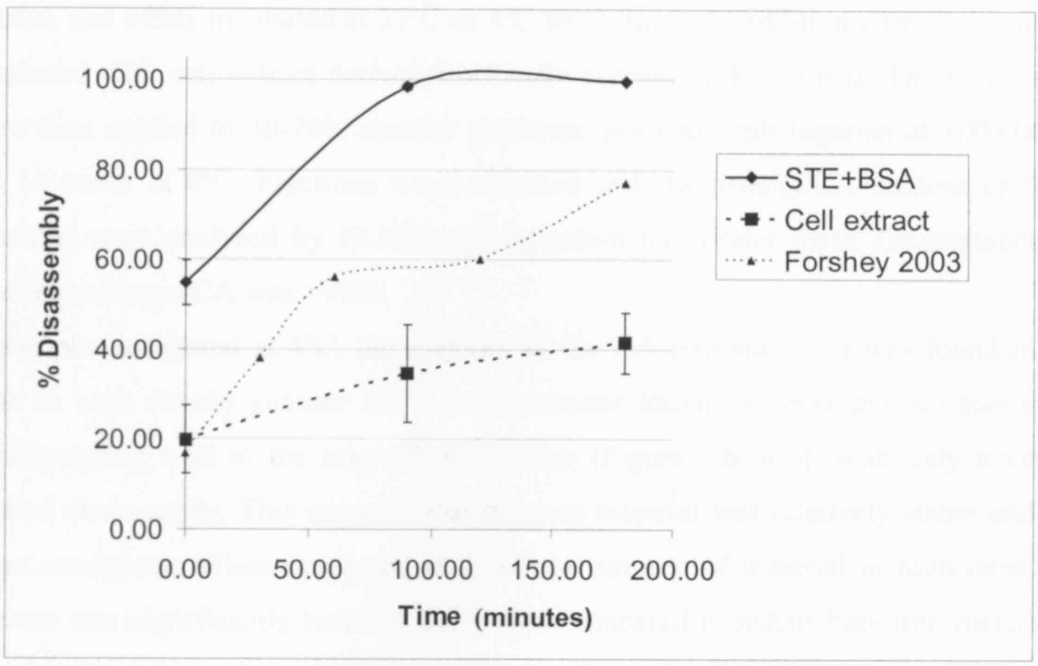


Figure 5.7 In-vitro disassembly of HIV cores.

HIV cores were incubated with STE BSA 10mg/ml or TE671 cell extracts at 37ºC. At the times indicated, samples were taken and subjected to centrifugation at 100000g. The amount of material in pellet and supernatant was determined by p24 ELISA. Percentage disassembly was calculated as amount in supernatant / (amount in pellet + amount in supernatant). Data from Forshey and Aiken 2003 is included for comparison.

To establish whether disassembly of HIV in such cell extracts does occur and to detect any effect of Trim5α upon it, HIV cores were mixed with non restricting cell extract, and either incubated at 37°C or 4°C for 3 hours. In addition cores were also incubated with cell extract derived from cells expressing RhTrim5α. The reactions were then applied to 30-70% sucrose gradients, prior to centrifugation at 100,000g for 16 hours at 4°C. Fractions were collected and the density/CA content of the fractions were analysed by ELISA and refractometry. Under these circumstances, recovery of input CA was >95%.

In samples incubated at 4°C, the majority of the CA (around 70%) was found in a peak in high density sucrose and a small amount found in lower density sucrose, corresponding well to the original preparation (Figure 5.6, top), with only a very limited disassembly. This suggests that the core material was relatively stable under these conditions. When incubated at 37°C, the amount of material in high density sucrose was significantly reduced and instead appeared to redistribute into material found at an intermediate density of sucrose, although notably without increase in material in low density sucrose, suggesting that complete disassembly into free CA does not occur (Figure 5.6, middle). There was no clear effect of Trim5α upon this process (Figure 5.6, bottom).

5.6 A disassembly assay for HIV core material

The data in the above experiment, whilst providing insight to the disassembly of HIV cores, are perhaps not appropriate for a disassembly assay where one must compare subtly different samples. Sucrose gradients when poured are inherently variable, as is the collection of fractions from them. In addition, the cost of such extensive ELISA based assays is considerable.

Another assay based simply upon the ratio of pelletable/free CA has been used to measure rates of HIV core disassembly. In this assay, cores are incubated in various conditions. At different time points samples are taken, subjected to centrifugation and the ratio of CA in the pellet compared to the supernatant is determined by CA ELISA. When this assay was employed, disassembly of HIV cores can be observed over time. Figure 5.7 shows the mean results from 3 such experiments.

In cell extracts, at time 0, 80% of CA is in a pelletable form. This is steadily reduced over 3 hours to around 60%.

When performed in STE buffer in the presence of 10mg/ml BSA, disassembly is very rapid although the effects of loss of CA to the reaction vessel are difficult to interpret. Even in the presence of 10mg/ml BSA and handling with blocked tips, the experiment shown in figure 5.3 suggested that 15% of material is lost, mainly from the pelleted fraction. On this basis, disassembly at t=0 could be closer to 20%. Despite this caveat, disassembly is clearly still much more rapid than in the presence of cell extracts.

Previous experiments using this assay with STE buffer have not reported this problem. Data from Forshey and Aiken is shown for comparison. In these experiments, significantly more CA material is used perhaps overcoming the loss of material present when only small quantities are used (the experiment here were designed with the aim of using as little material as possible in order to prevent any saturation of restriction factor). Under those circumstances, Forshey also observed much more rapid disassembly in STE buffer than observed in the cell extracts shown here with only 20% in a pelletable form after 3 hours. (Cell extracts were not tested in the experiments of Forshey). Taken together, these data suggested that the presence of cell extracts could have a stabilising effect upon HIV cores.

5.7 Protein components of human cell extracts act to stabilse HIV CA cores

In the absence of cell extracts, HIV disassembly rates have been shown to be dependent upon a number of factors including pH and salt concentration, with cores particularly stable in high pH and high salt (Forshey and Aiken, 2003). It seemed possible that simply pH or salt conditions in cell extracts may favour core stability relative to experiments performed in STE buffer alone. Alternatively, protein factors may regulate core disassembly in the cytoplasm. In order to better discriminate between these two possibilities, cell extracts were subjected to centrifugation through molecular weight filters of 5kDa, 10kDa and 50kDa size. The filtrates were then used in disassembly assays and compared to whole cell extract (Figure 5.8). In these conditions, the use of smaller molecular weight cut off filters enhanced rates of CA

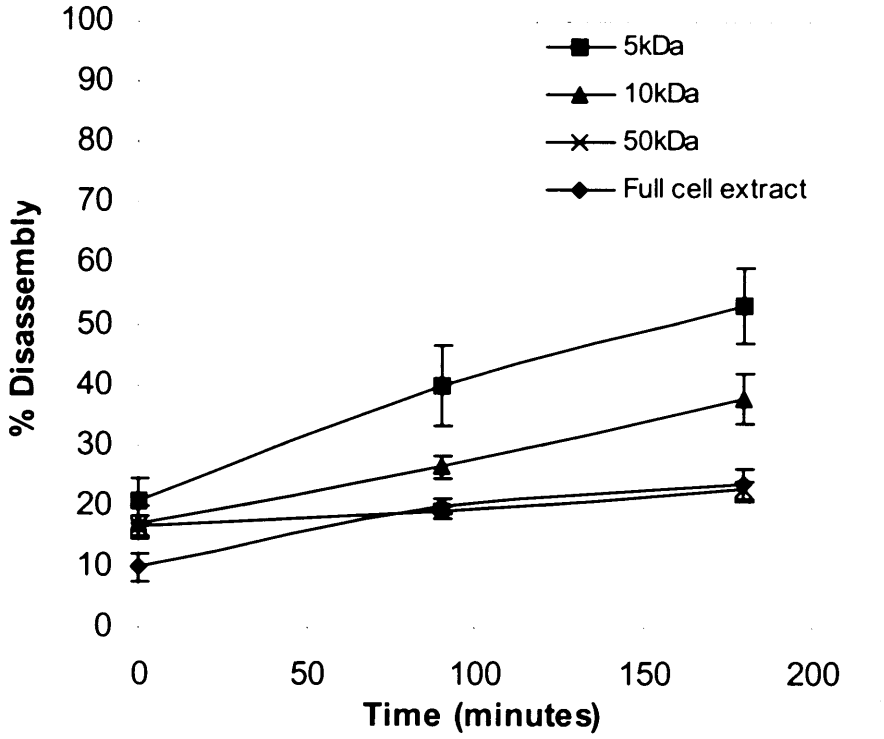


Figure 5.8 In-vitro disassembly of HIV cores in filtered cell extracts
HIV cores were incubated in the presence of cell extracts filtered through 5kDa, 10kDa and 50kDa filters. Samples were taken at the indicated time points and the percentage disassembly determined by centrifugation followed by quantitation of CA in pellet and supernatant. Data are the mean of 3 independent experiments. Error bars show +/- standard deviation.

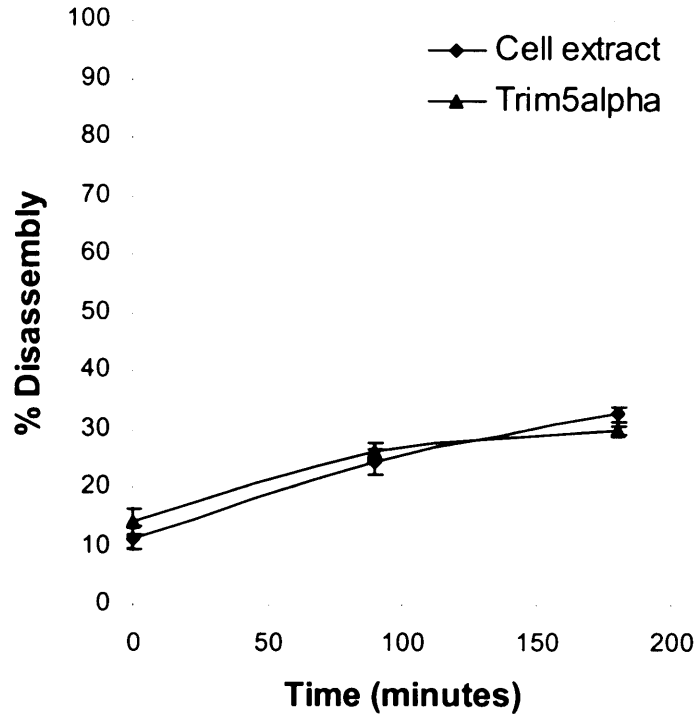


Figure 5.9 In vitro disassembly of HIV cores in the presence of Rhesus Macaque Trim5α.
The disassembly assay was performed in extracts derived from wild type TE671 cells or cells stably expressing Trim5α. Samples were taken at the indicated time points and percentage disassembly determined following centrifugation by quantitation of CA in pellet and supernatant by ELISA. Data shown are the mean of 3 independent experiments. Error bars show standard deviation.

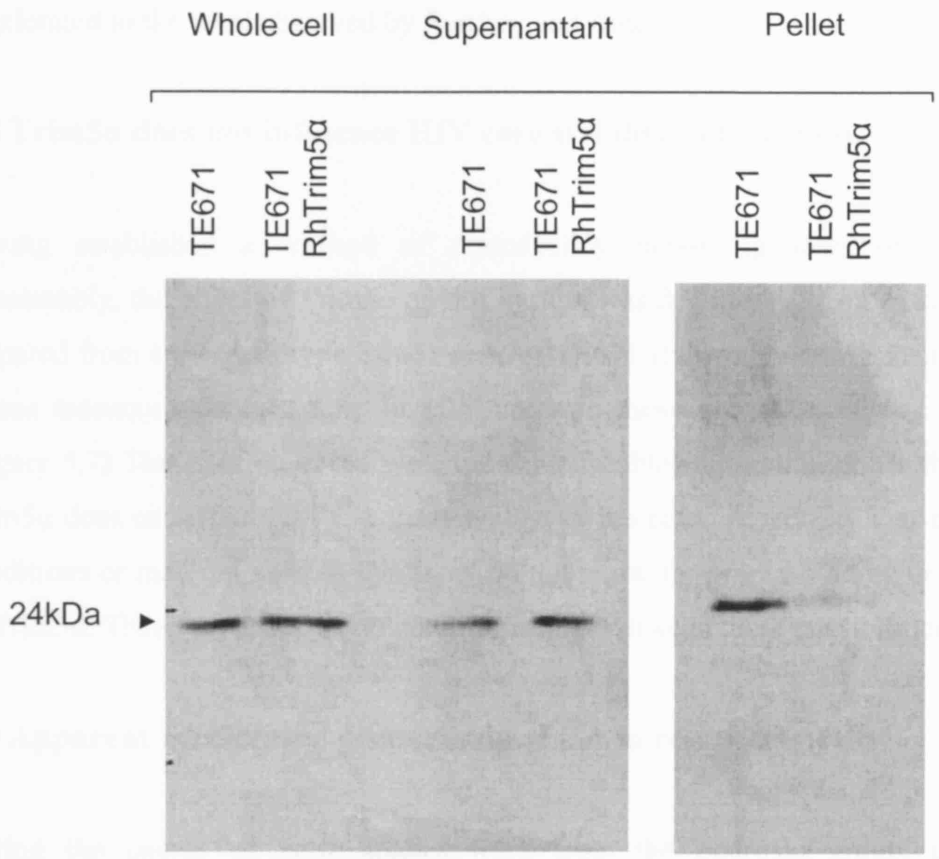


Figure 5.10 Analysis of intracellular HIV CA material from restricting and non-restricting cells
$3x10^6$ TE671 cells were infected with 10ml of HIV containing 293T supernatant for 14 hours. Cells were lysed by homogenisation and cleared by low speed centrifugation to remove large debris. A sample of this material was taken (Whole cell extract, lanes 1,2). The remaining material was placed onto a 50% sucrose cushion and centrifuged to separate cytosolic cores from endosomal CA and free CA. Samples of pellets and supernatants were analysed by SDS-PAGE and western blot for CA.

disassembly, suggesting that high molecular weight molecules can act to stabilise HIV cores in conditions of equivalent pH and salt. In these circumstances, all recovery rates were > 95%. Using the smallest cut off, disassembly rates were accelerated to the level observed by Forshey and Aiken.

5.8 Trim5α does not influence HIV core stability in this assay

Having established a method of reproducibly measuring rates of HIV CA disassembly, the effect of Trim5α on this system was determined. Cell extracts were prepared from either wildtype TE671 cells or TE671 stably expressing Trim5α from rhesus macaques. Disassembly of HIV cores in these cell extracts was analysed (Figure 5.7) The rates observed were indistinguishable, suggesting either that either Trim5α does not affect HIV CA disassembly, or the equal possibility that either the conditions or material used in this assay do not allow for proper binding or function of Trim5α. These experiments do not discriminate between these possibilities.

5.9 Apparent accelerated disassembly of CA in restricting cells

During the course of these studies work from the Sodroski group, using an alternative *in vivo* approach, suggested that Trim5α can cause accelerated un-coating of HIV and MLV. In this assay, cells are infected with virus, lysed at various time points and the lysates centrifuged through a 50% cushion sucrose to pellet cytoplasmic core material, separating it from free CA and endosomal associated material which remains at the interface between 50% sucrose and cell extract. The amount of pelletable CA in this system is determined by western blot, as is the amount of non-pelletable CA by removing the top 100μl of extract. The authors observe that in restricting cells less pelletable CA is found, and suggest that in the case of MLV at least, this corresponds with an increase in free CA.

In order to confirm these data and establish their importance, the assay was implemented and the experiments reproduced. Figure 5.9 shows the outcome of such an experiment. 10ml of HIV containing supernatants were used to infect wild type TE671 cells or TE671 cells expressing Trim5α for 14 hours. Cells were lysed by homogenisation and cell debris removed by low speed centrifugation. Samples were

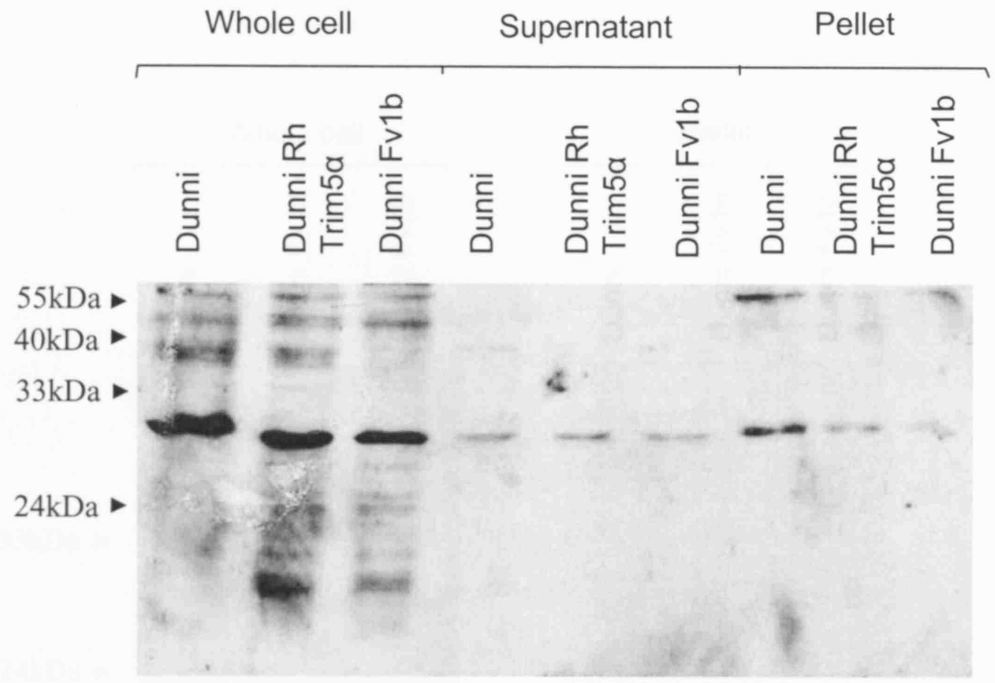


Figure 5.11 Analysis of intracellular MLV CA material from restricting and non-restricting cells

$3x10^6$ *M.dunni* cells, wild type or expressing RhTrim5α or $Fv1^b$ were infected for 14 hours with 10ml of N-tropic MLV containing supernatant from a chronically infected cell line. Cells were lysed by homogenisation and cleared by low speed centrifugation to remove large debris. A sample of this material was taken (Whole cell extract, lanes 1,2). The remaining material was placed onto a 50% sucrose cushion and centrifuged to separate cytosolic cores from endosomal CA and free CA. Samples of pellets and supernatants were analysed by SDS-PAGE and western blot for CA.

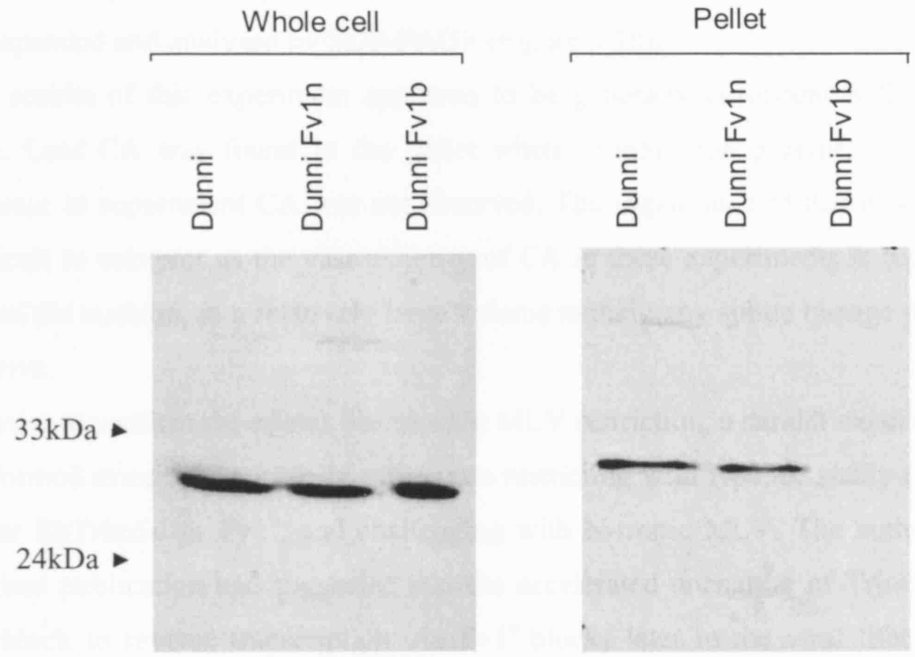


Figure 5.12 Analysis of intracellular N-MLV CA material from cells expressing $Fv1^n$ and $Fv1^b$
$3x10^6$ *M.dunni* cells, wild type or expressing $Fv1^n$ or $Fv1^b$ were infected for 14 hours with 10ml of N-tropic MLV containing supernatant from a chronically infected cell line. Cells were lysed by homogenisation and cleared by low speed centrifugation to remove large debris. A sample of this material was taken (Whole cell extract, lanes 1,2). The remaining material was placed onto a 50% sucrose cushion and centrifuged to separate cytosolic cores from endosomal CA and free CA. Samples of pellets were analysed by SDS-PAGE and western blot for CA.

taken of the resultant lysates before application to a 50% sucrose cushion and centrifugation at high speed. After centrifugation, a sample from the top of the tube was taken prior to aspiration of the entire supernatant. The pellet was then resuspended and analysed by SDS-PAGE (Figure 5.10).

The results of this experiment appeared to be generally consistent with published data. Less CA was found in the pellet when Trim5α was present. A significant increase in supernatant CA was not observed. The importance of this observation is difficult to interpret as the vast majority of CA in these experiments is found at the top of the cushion, in a relatively large volume making any subtle change difficult to observe.

In order to confirm the effects observed in MLV restriction, a similar experiment was performed using *M.dunni* cells, either non restricting wild type, or stably expressing either RhTrim5α or $Fv1^b$, and challenging with N-tropic MLV. The authors of the original publication had suggested that the accelerated uncoating of Trim5α caused the block to reverse transcription. As $Fv1^b$ blocks later in the viral lifecycle after reverse transcription, it was included to test this assumption.

Testing these cells lines with N-tropic MLV, Trim5α did indeed appear to reduce the amount of pelletable material, confirming the original data, although again an increase in free material was not apparent (Figure 5.11). Surprisingly, $Fv1^b$ also had a similar effect. In order to confirm that this effect was specific to $Fv1^b$ for N-tropic MLV, a similar experiment was performed for $Fv1^n$ which does not restrict N-tropic MLV. In this case, no effect on pelletable CA was observed (Figure 5.12).

These data suggest that as determined by this assay, the amount of pelletable CA is reduced by Trim5α and $Fv1^b$ suggesting that both restriction factors, despite blocking N-MLV at different stages of the life cycle can lead to a similar fate for the virus.

5.10 Discussion

This chapter has described experiments designed to test the hypothesis that Trim5α can affect the disassembly or stability of HIV-1 cores. HIV-1 cores were produced and incubated with cell extracts and the process of disassembly was followed. HIV-1 cores were found to be extremely stable in cell extracts, when compared to other experiments using a similar assay. A significant amount of material was still in

pelletable form after 3 hours of incubation at 37°C. This contrasts starkly with previously published data suggesting that HIV cores lose CA extremely rapidly after entering the cytoplasm.

A number of factors have been shown to influence core stability, including pH and Na^+ concentration. In order to better determine the cause of this apparent stability observed here, preserving pH and salt concentration, cell extracts were passed through molecular weight filters of various sizes. Using a very low cut off molecular weight filter (5kDa), core stability was reduced significantly, with a larger filter pore conferring greater stability (10kDa), up to that of wildtype when using a 50kDa cut off. Such filters are of course imperfect, with shape, charge and association in larger complexes affecting passage. The pore size values given by the manufacturer are therefore only approximate and would be unlikely to allow passage of any large complexes. Despite this, it does appear that allowing increasingly larger protein components into the filtrate does enhance stability of HIV-1 cores. It will be important to determine specifically which proteins are responsible.

Having established an assay to follow core disassembly in cell extract, the effect of Trim5α on this system was determined. No effect could be observed on disassembly or indeed any binding of restriction factor shown. Possible explanations for this lack of effect are discussed in Chapter 8.

Using an *in vivo* assay, both Trim5α and Fv1 could be shown to reduce the amount of pelletable CA for both HIV-1 and MLV suggesting that accelerated disassembly may in fact occur. Contrary to previously published data, little increase in free CA was observed. Analysing the amount of free CA present is complex due to the design of the experiment where the 'top 100μl' are removed from above the cushion. Given that the cell extract extends for 2ml above the cushion, composing endosomal material as well as free capsid, defining this 'top 100μl' and removing it reproducibly is challenging.

The experiments here describe an *in vitro* and an *in vivo* approach for examining the disassembly of HIV cores in the presence of restriction factors, each producing contradictory results. Possible implications for the mechanism of action of restriction factors are discussed in detail in Chapter 8.

CHAPTER 6

The Importance of Hexamer Lattice Contacts Mediated by the N-terminal Domain of Capsid in the Formation and Stability of the MLV and HIV-1 Virions

Cryo-electron microscopy reconstructions have shown that retroviral capsid assemblies are made up of a lattice of hexamers. Several regions of HIV CA have been suggested as important for assembly of CA into such polymeric structures. Those in the NTD are located in positions which would likely make contacts to adjacent subunits within the hexamer. Mutations which disrupt assembly can also be made in the CTD of CA, most focused around a putative hydrophobic dimer interface. The CTD may be involved in dimeric contacts between adjacent hexamers. The role of the NTD in such hexamer-hexamer contacts is unclear in either the immature or mature form.

Recently, a crystal structure of the MLV CA NTD was determined (Mortuza et al., 2004). The repeating unit within these crystals was found to be a hexamer. Analysis of interactions between monomers within this hexamer has revealed important information about the structure of the capsid shell.

As well as structural data on the monomer and hexamer, a third level of information was also available - hexamers were found in planar layers (Figure 6.1). Within these layers, trimeric contacts were observed between hexamers, with each capsid subunit contacting two others in adjacent hexamers (Figure 6.2, Top). Closer examination of this region revealed a network of H-bond interactions between positively charged Asp and negatively charged Arg residues within the trimer (Figure 6.2, bottom).

The previous chapters have ascribed considerable importance to the structure and possibly the stability of the polymeric CA core. An explanation of the inter-molecular interactions which form and maintain it may well be critical to obtaining insight into the mechanism of restriction factors, as well as leading to a better understanding the role of the CA protein in the life-cycle of the virus. To this end, investigations began in close collaboration with Dr. Ian Taylor and Dr. Nehar Mortuza (NIMR Division of Protein Structure) to examine the biological relevance o

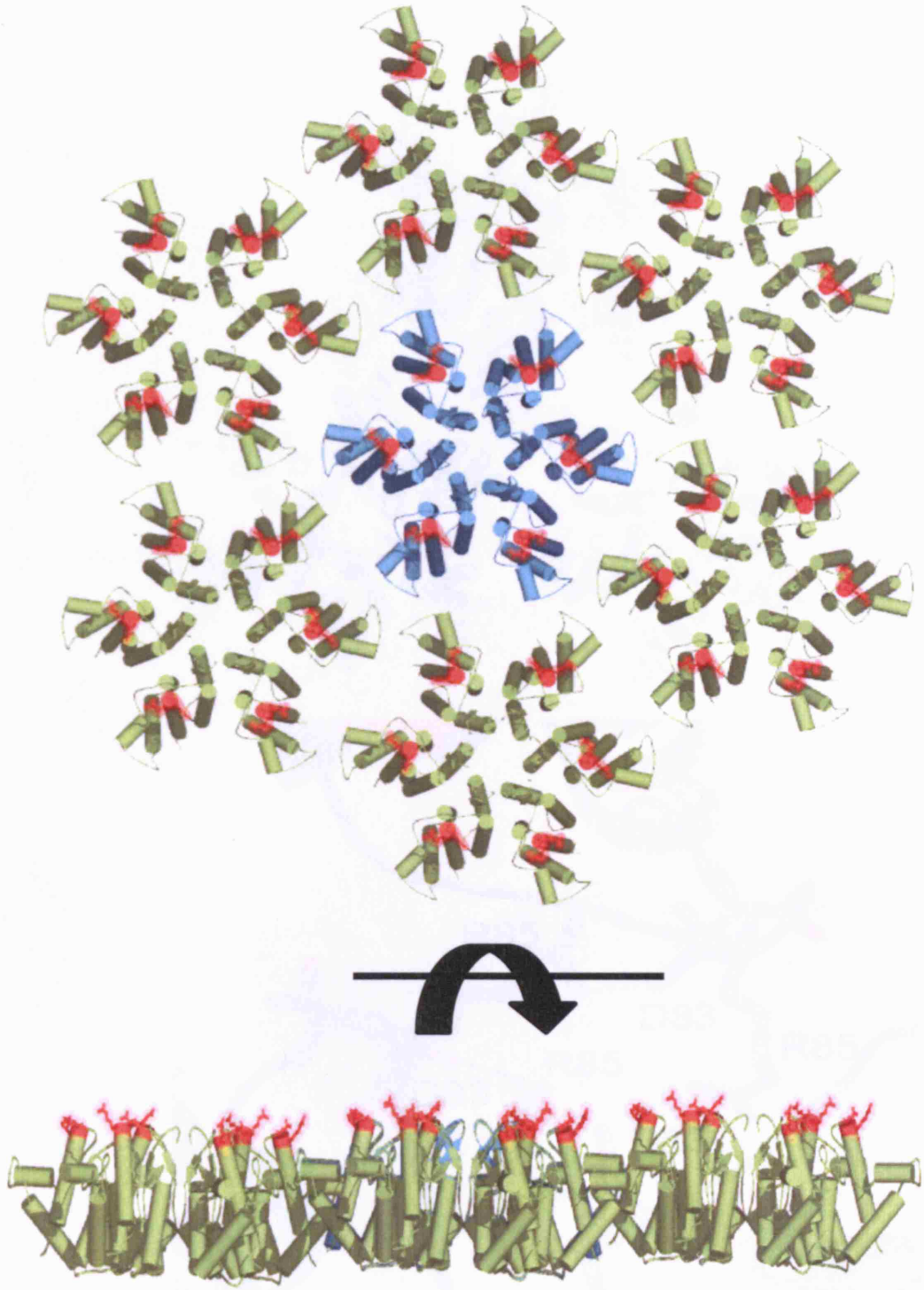


Figure 6.1 Lattice arrangement in crystals of the N-terminal domain of N-tropic Murine Leukemia Virus capsid protein.
The NTD of MLV CA was found within its crystals in the form of hexamers. Hexamers were arranged in planar sheets with trimeric contacts between the hexamers.
For reference, CA 110, shown to be important in virus restriction by Fv1, and on the outer surface of the core, is shown in red. (Data supplied by Dr. Nehar Mortuza, NIMR Division of Protein Structure).

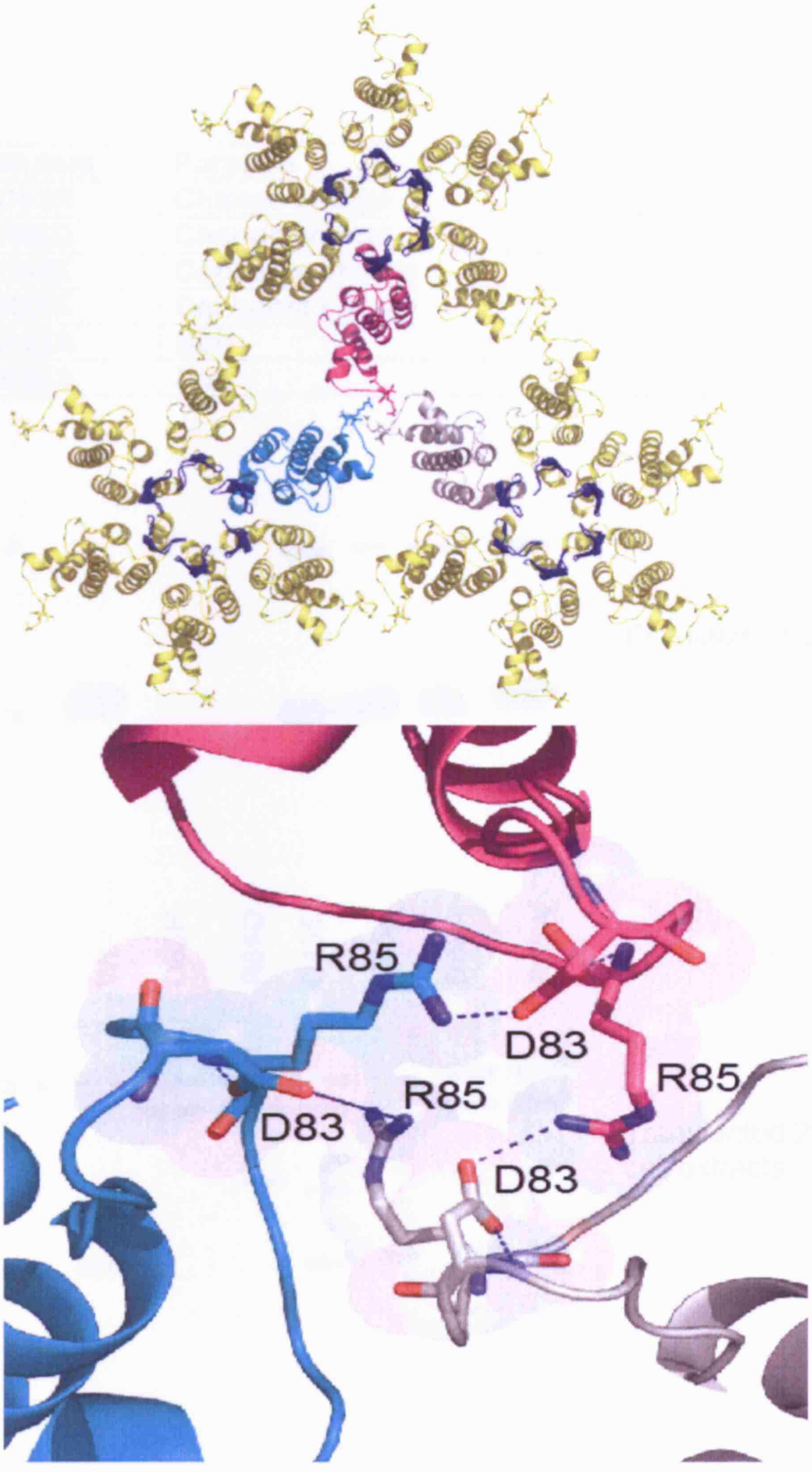
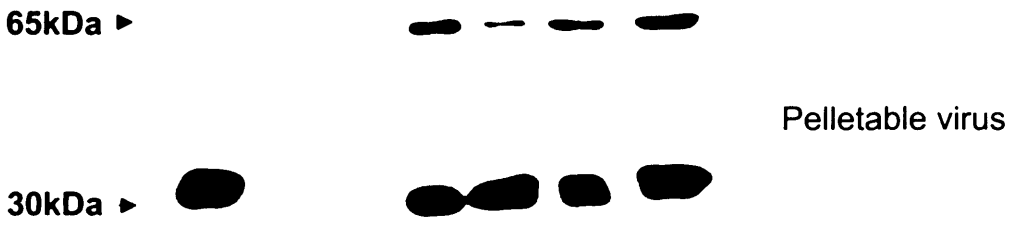


Figure 6.2 Inter-hexamer contacts found within crystals of the NTD domain of CA from MLV. Trimeric interface between subunits (Top). Specific hydrogen bonds observed between D83 and R85 in adjacent molecules.(Data supplied by Dr. Nehar Mortuza, NIMR Division of Protein Structure).

A

Mutant	Purpose
D83R	Change charge
R85D	Change charge
D83E	Conserve charge
R85K	Conserve charge
D83A	Null
R85A	Null

B



C

Figure 6.3 MLV capsid mutants designed to disrupt potential hydrogen bonds in the trimeric interface between hexamers.
(a) Table showing mutations introduced into CA and their intended purpose. (b) Western blot probed with anti-CA monoclonal antibodies showing amounts of pelletable virus obtained 2 days post transfection of Gag-Pol expression constructs. (c) Western blot showing CA in cell lysates from the same cells.

f the hexamer-hexamer contacts observed within MLV CA N-terminal domain crystals. This chapter describes the outcome of these experiments.

6.1 CA mutations designed to disrupt charge interactions at the trimer interface

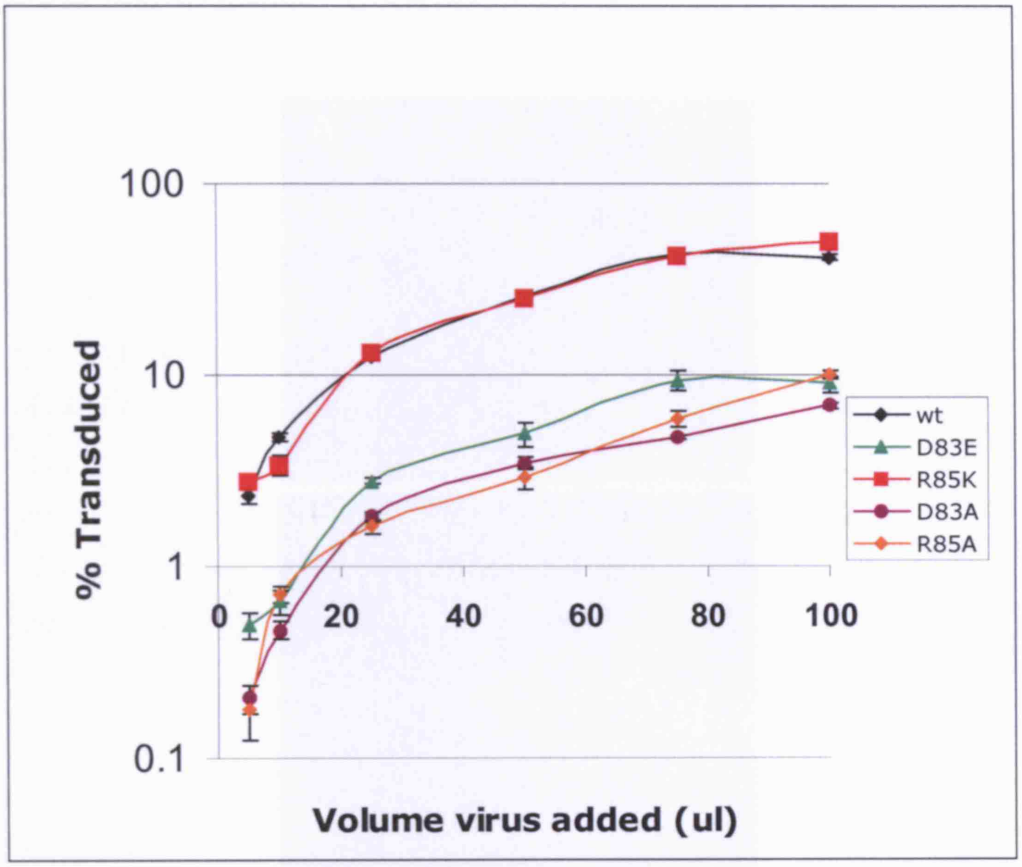
In order to examine the importance of D83 and R85 interactions in MLV CA assembly, a series of mutations were introduced into CA at these positions in Gag-Pol expression constructs. These mutations are described in Figure 6.3. Single mutations were made to either conserve the charge on the residue (D83E, R85K), remove the side chain (D83A, R85A) or change the charge (D83R, R85D).

Cells were transfected with these constructs as well as plasmids encoding VSV-G and a vector containing eGFP. In order to determine any effects on virus production, virions were pelleted by centrifugation and analysed by western blot probing for CA. Such an analysis revealed that the D83R and R85D mutations entirely blocked particle production whereas mutations which either conserved the charge or removed the side chain had little effect on particle production (Figure 6.3), showing similar amounts of processed CA, although with some accumulation of unprocessed Gag. A double mutant D83R R85D showed an identical phenotype to that of the single mutations.

In order to confirm that the D83R and R85D mutations did not impair Gag translation or stability, transfected 293T cells were lysed and the extracts examined by western blot for CA. In cells expressing wildtype MLV, a band of 65kDa was seen as well as a band at 30kDa. The band at 65kDa corresponds to full length Gag. The band at 30kDa is processed CA. Whether this band represents some intracellular Gag processing, virions still associated with the plasma membrane, budding into an intracellular compartment or re-infection of the cells is not at all clear. It should however be noted that this band is absent from the two mutants which do not produce particles. In these two mutants, levels of 65kDa Gag are increased along with a smaller product at around 55kDa. For both mutants, another band is seen at around 40kDa, with the band in the R85D mutant slightly larger and more intense.

The conservative and alanine substitution mutants had a band at 30kDa although this band was slightly less intense than wildtype MLV. All but R85K showed an increase

A



B

Mutation	Approximate infectivity relative to wild type
D83R	N/A – no virus
D83E	0.16
D83A	0.12
R85D	N/A – no virus
R85K	No Effect
R85A	0.11

Figure 6.4 Infectivity of MLV CA mutants
The MLV CA mutants from figure 6.3 carrying an eGFP vector were used to infect $4x10^4$ *M.dunni* cells plated in 12 well plates 24 hours previosuly. Increasing volumes of virus were added to cels. Percentage transduced cells was determined by flow cytometry for GFP (a) Values shown are mean of 2 experiments. Error bars show standard deviation.
The ratio of percentage transduced cells at 25µl and 50µl were used to determine the relative infectivity when compared to wildtype (b).

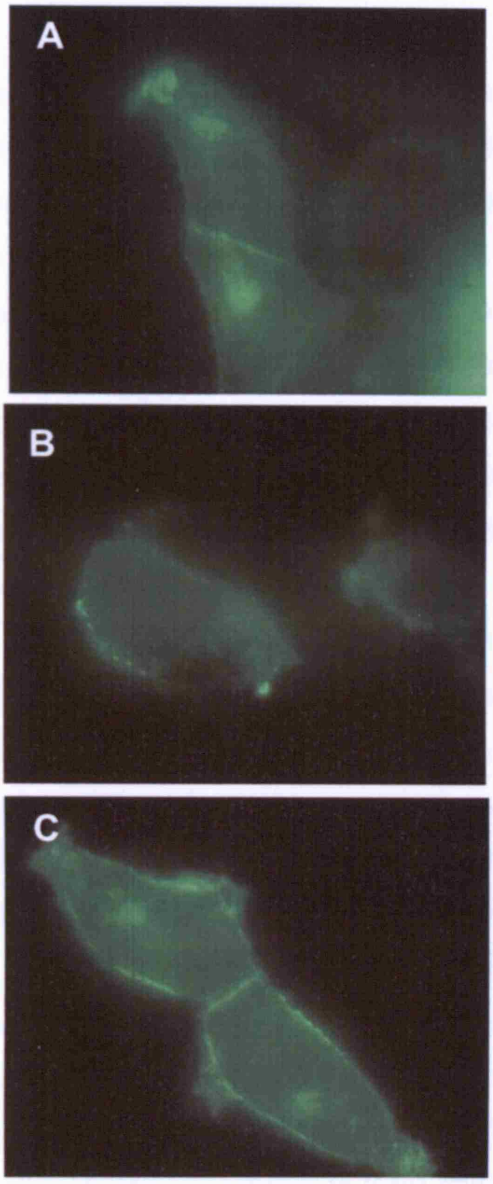


Figure 6.5 Immuno-fluorescence image showing localisation of Gag in CA mutants.
293T cells transfected with Gag-Pol expression constructs expressing either Wt (a), D83R (b) or R85D (c). After 2 days, cells were fixed with paraformaldehyde then probed with a monoclonal antibody directed against CA. This was detected with a secondary antibody conjugated to AlexaFluor 488.

in 65kDa material as well as the other bands observed in the charge disruption mutants. These data indicate that mutations which reversed the charge on either D83 or R85 block particle production and lead to an increased amount of unprocessed Gag associated with the cell.

Despite not affecting particle production, less drastic mutations do appear to have some effects on the behaviour of Gag in these transfected cells. All except R85K showed more unprocessed Gag as well as lower weight bands, suggesting that these mutations may have some phenotypic consequences.

In order to examine these effects more closely, the relative infectivity of these particles was determined by titration on *M.dunni* cells. The percentage of transduced cells was quantified by flow cytometry (Figure 6.4). The conservative mutation at position D83 results in an infectivity around 20% compared to wildtype, with the alanine substitution at this position causing an infectiviy of around 12.5%. The conservative mutation in R85 had no effect on infectivity whilst the alanine mutation reduced infectivity by around 11% of wildtype. These results correlate reasonably well with the cell lysate western blot in that the mutations producing an abnormal phenotype there also impacted upon infectivity whilst the R85K mutant did not. There is also an indication of a progressive severity of phenotype corresponding with conservative mutation, alanine substitution and change of charge respectively.

The D83R and R85D mutations clearly block particle production. In order to better define the stage of the block, 293T cells transfected with either wildtype or mutant Gag-Pol constructs were examined by immunofluoresence for CA (Figure 6.5). Wildtype material showed some intracellular peri-nuclear staining as well as material in the cell membrane. Both D83R and R85D showed a similar pattern, indicating that there is no overt change in gag localisation in these mutants. Importantly, it is clear that Gag had no difficulty in reaching the plasma membrane suggesting that the block in particle production may be in proper Gag assembly.

In an attempt to further define this block, transfected cells were examined by thin section transmission electron microscopy. In wildtype cells, both immature and mature extracellular particles were readily observed, some in the process of budding from the cell membrane. Consistent with western blot data, no extracellular particles were observed for either the D83R or R85D mutant (Figure 6.6, a)

Previous reports have indicated that mutations which block assembly can lead to intermediate structures forming in the plasma membrane. For the D83R mutant, such

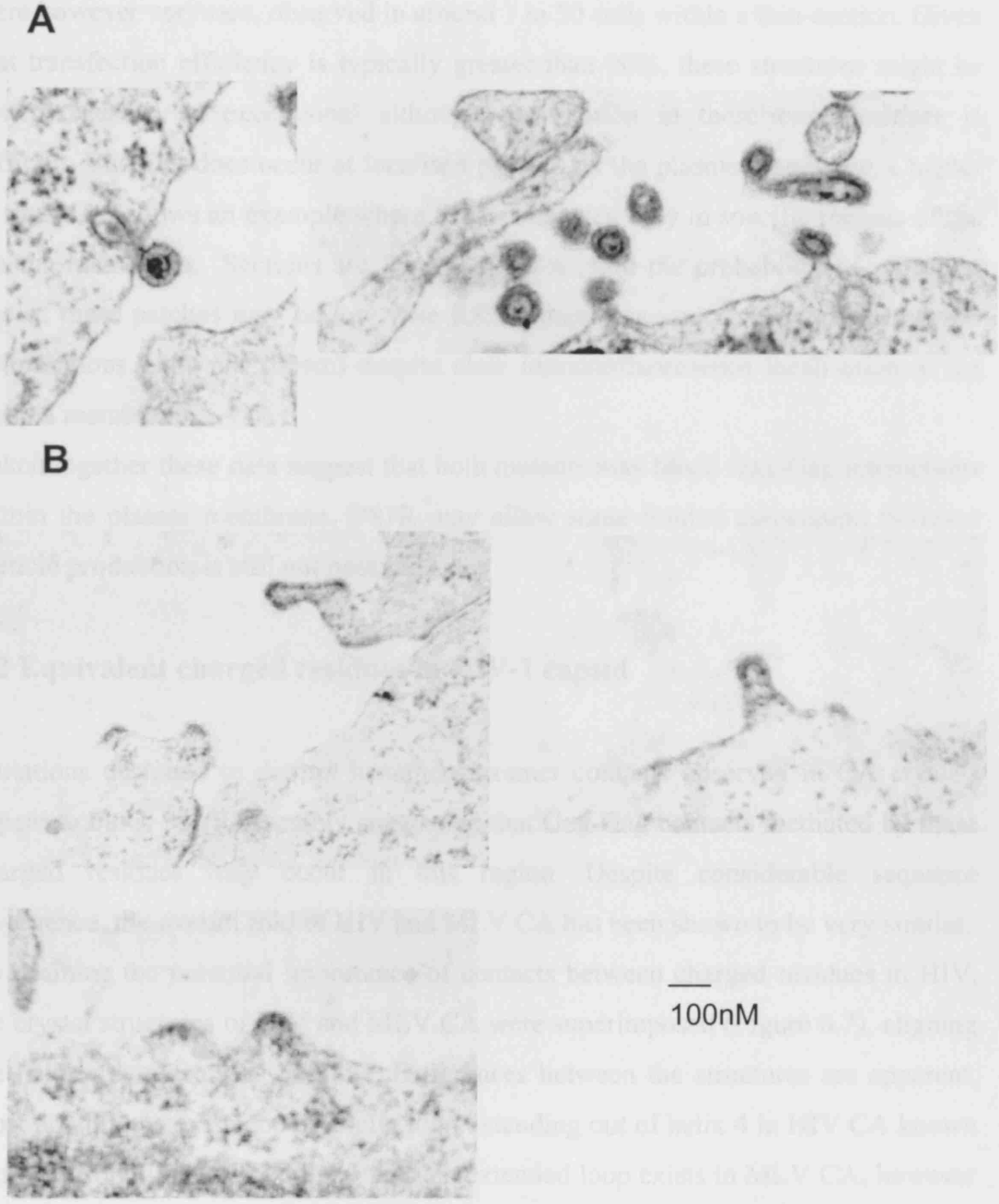


Figure 6.6 Thin section electron micrographs of cells transfected with MLV Gag-pol expressing constructs. Cells were fixed with glutaraldehyde/paraformaldehyde and stained with uranyl acetate prior to analysis.

(a) Wildtype – Budded virions and those in the process of budding are visible
(b) D83R – membrane deformations are occasionally observed with D83R mutant

structures were occasionally observed as electron dense deformations in the membrane, not observed in non-transfected cells (Figure 6.6, b) Such deformations were however very rare, observed in around 1 in 50 cells within a thin-section. Given that transfection efficiency is typically greater than 90%, these structures might be better regarded as exceptional although quantitation in these circumstances is difficult. Budding does occur at localised patches on the plasma membrane. Chapter 4, figure 4.2 shows an example where p12 is observed only in specific regions of the plasma membrane. Sections are 50nM thick therefore the probability of capturing one of these patches may be low. The R85D mutant caused no obvious membrane deformations (data not shown) despite clear immunofluoresence localisation to the plasma membrane.

Taken together these data suggest that both mutants may block Gag-Gag interactions within the plasma membrane. D83R may allow some limited association however particle production is still not possible.

6.2 Equivalent charged residues in HIV-1 capsid

Mutations designed to disrupt hexamer-hexamer contacts observed in CA crystals appear to block MLV assembly suggesting that Gag-Gag contacts mediated by these charged residues may occur in this region. Despite considerable sequence divergence, the overall fold of HIV and MLV CA has been shown to be very similar.

To examine the potential importance of contacts between charged residues in HIV, the crystal structures of HIV and MLV CA were superimposed (Figure 6.7), aligning the helices as closely as possible. Differences between the structures are apparent, most notably the existence of a long loop extending out of helix 4 in HIV CA known as the cyclophilin binding loop. No such extended loop exists in MLV CA, however a small lateral loop does exist after helix 4, upon which D83 and R85 are found (Figure 6.7).

In the equivalent region in HIV, charged residues are also present, E79 and R82, could potentially make similar contacts to those observed in the MLV structure. E79 is found on the tip of helix 4 whereas R82 sits at the base of the cyclophilin binding loop. Both residues project outwards, potentially available to make inter-molecular contacts.

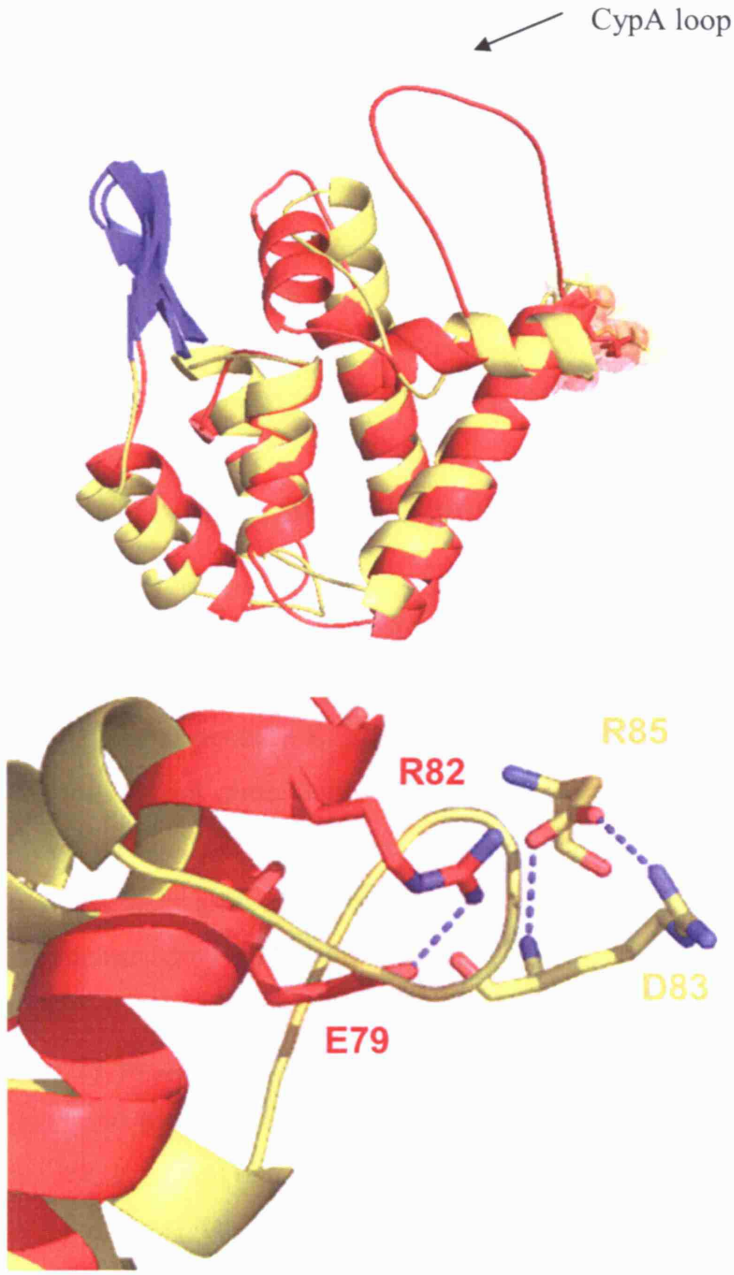


Figure 6.7 Structural alignments of HIV (red) and MLV (yellow) CA based on published crystal structures. In MLV, D83 and R85 are found on a loop above helix 4 and project outwards. Equivalently charged residues exist in HIV although not on such an extended loop, instead forming part of helix 4, but still projecting outwards potentially available to make contacts with ontact adjacent molecules.
Model data supplied by N. Mortuza.

Mutations to alanine have previously been made at both of these positions resulting in a moderate drop in infectivity but with little other phenotype described. A leucine mutant at position 82 has been described as 'aberrant: multiple p24 species observed', again with a modest drop in infectivity (Owens et al., 2004). The importance of this observation is not clear.

In order to further characterise any contacts made by these residues, a similar mutagenesis approach was adopted to that used with MLV.

6.3 E79R and R82D mutation In HIV-1 capsid

To examine the potential importance of E79 and R82 in HIV-1 CA interactions, the charge at either position was swapped by changing the glutamate at position 79 to arginine and the arginine at position 82 to aspartate in Gag-Pol expression constructs. Constructs were transfected along with VSV-G and a vector carrying eGFP into 293T cells. Particle production and cell extracts were analysed by western blotting for CA using a monoclonal antibody (Figure 6.8). No difference was observed in the cell extracts. As measured by p24 levels, particles were produced in approximately equal amounts. E79R was indistinguishable from wildtype. R82D showed similar amounts of properly processed CA in addition to smaller amount of various p24 products of higher and lower molecular weight, in concordance with that previously described for the R82L mutant (Owens et al., 2004).

The phenotype for these HIV mutants is clearly different to that observed for MLV in that particles are produced in equivalent amounts. In order to examine any effects on infectivity, viruses were titrated on TE671 cells with the percentage transduced cells determined by flow cytometry. The impact on infectivity was dramatic, with the R82D infectivity reduced infectivity to around 2% of wildtype and the E79R mutant around 1% (Figure 6.9), In order to better characterise this loss of infectivity, levels of early reverse transcription products in newly infected cells were analysed by quantitative PCR. Early RT products were found to be reduced to around 15% of wildtype for R82D and 10% for E79R suggesting that an important factor in the loss of infectivity is a loss in the ability to properly initiate reverse transcription (Figure 6.10a). To determine whether the E79R and R82D mutants possessed an intrinsic deficiency in initiating reverse transcription (perhaps due to aberrant RNA or RT

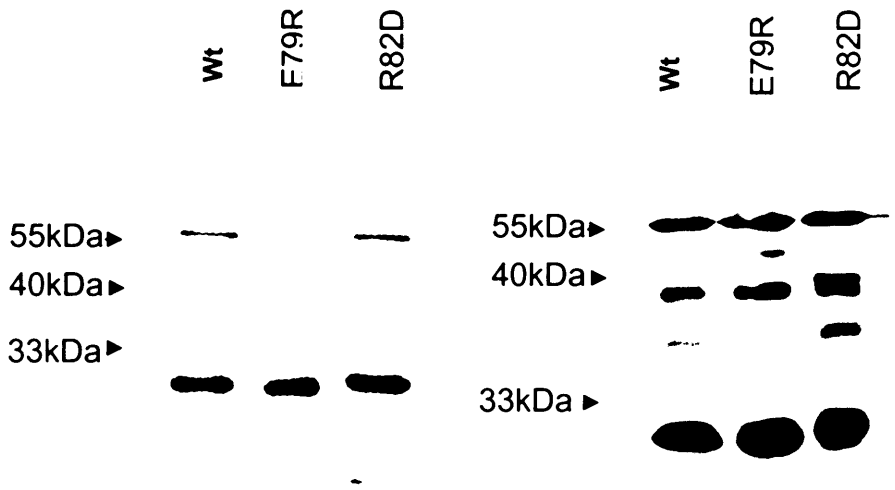


Figure 6.8 Western blots showing particle production and expression in cells of HIV CA mutants E79R and R82D.

293T cells were transfected with Gag-Pol expression constructs of either wildtype, E79R or R82D. Samples were separated by SDS-PAGE prior to transfer to PVDF membranes and analysis by western blot. Membranes were probed with a monoclonal antibody directed against HIV CA (a) Viral containing 293T supernatant pelleted through 20% sucrose (b) Cell lysates from transfected cells.

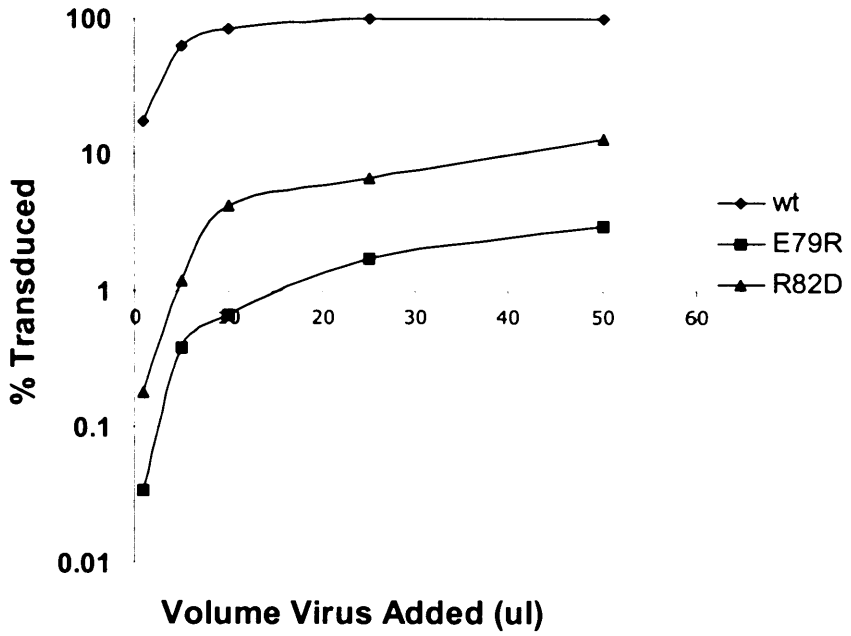


Figure 6.9 Infectivity of HIV CA mutants. Viruses carrying a GFP vector were added to $4x10^4$ TE671 cells plated in 12 well plates 24 hours previously. Viruses were added in increasing volumes. Cells were incubated for 2 days post infection to allow expression of GFP and the percentage of transduced cells was determined by flow cytometry.

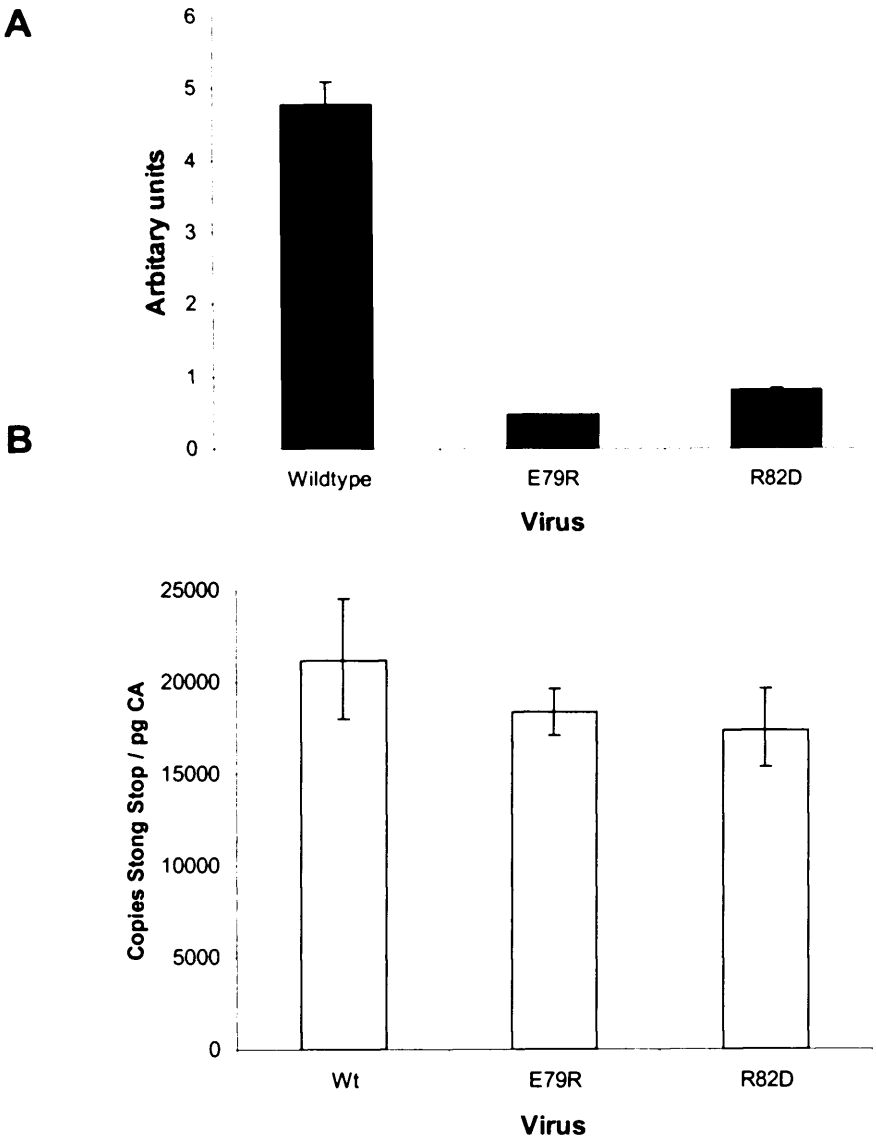


Figure 6.10 Quantitative PCR analysis to examine RT potential of wildtype HIV and E79R/R82D mutants.
(a) Early RT products in cells. Cells were infected with equal quantities of virus (as determined by western blot for CA). After 6 hours, cells were lysed and DNA purified. Quantitative PCR was performed probing for early products of reverse transcription (R-U5, strong stop DNA – see *Materials and Methods*). Amounts shown are arbitrary units for comparison.
(b) ERT reactions. Virus preparation was incubated with 0.02% NP-40, 2.5mM $MgCl_2$ and 5mM dNTPs for 4 hours at 37°C. DNA was purified and Quantitative PCR was performed as above to determine copies of strong stop DNA. Amount of CA was determined by ELISA. Error bars show standard deviation from 5 replicates in 1 experiment.

incorporation) or whether the mutants disrupted a function of CA required for infection of a cell, endogenous reverse transcription (ERT) assays were performed. In these assays, virions are incubated at 37°C with dNTPs and a small quantity of detergent to partially permeabilise them. DNase I is added after the incubation to remove any DNA not present in intact virions or carried over from transfection. DNase I is inactivated, the virion DNA isolated, and any remaining plasmid DNA is removed by digestion with DpnI. The amount of DNA present is then determined by quantitative PCR. In such experiments, the ERT reaction resulted in around a 10-fold increase in strong stop DNA compared with a control reaction where dNTPs were not supplied and the mixture incubated at 4°C. The mutants showed an equivalent increase in early RT products (Figure 6.10b). In addition, absolute levels (around 20,000 copies per pg CA) of RT product produced by wild-type or mutant virus were similar, indicating that the mutants are fully capable of reverse transcription. These experiments suggest that the E79R and R82D mutations do not directly affect the viral RNA or reverse transcriptase, but rather disrupt a function of CA in the early stages of the infection of a cell.

The ability to initiate reverse transcription within the cell has been linked to possession of a mature CA core of proper stability (Forshey et al., 2002; Tang et al., 2003). A series of experiments were therefore undertaken to examine whether the E79R or R82D mutations affected core stability or composition with the aim of shedding light on the importance of this region.

6.4 Abrogation of restriction by E79R and R82D

The experiments described in chapters 3 and 4 attach considerable importance to the existence of a mature MLV CA core in the abrogation of restriction by Trim5α and Fv1. In addition, work by the Aiken group has suggested that an HIV-1 core of optimum stability is necessary for the abrogation of Trim5CypA restriction in Owl Monkey cells (Forshey et al., 2005). If the mutations significantly affected the stability of the HIV capsid core, they might be predicted to also affect the abrogation properties of the virus, therefore the ability of E79R and R82D to abrogate Trim5α and Trim5CypA restriction was tested.

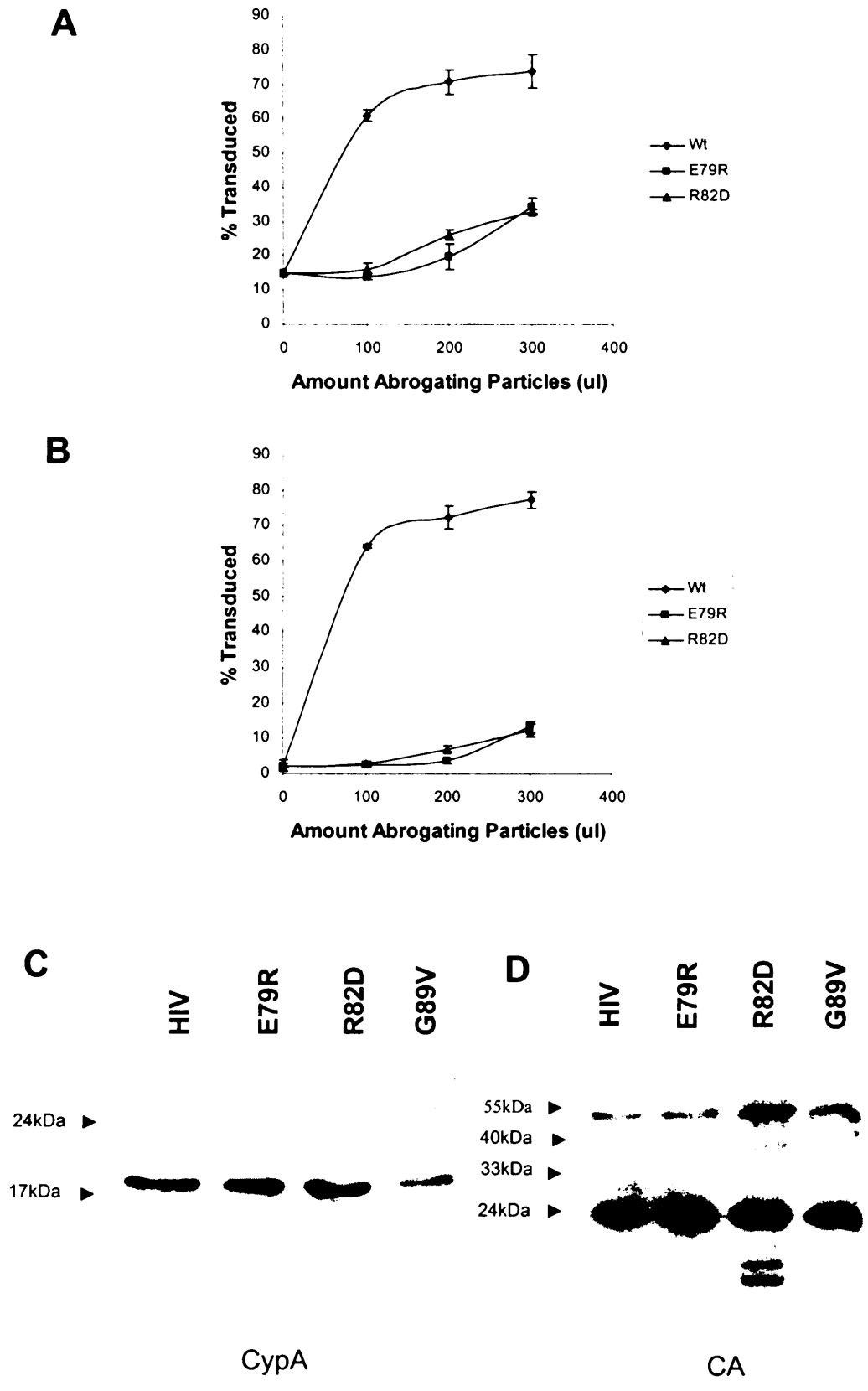


Figure 6.11 Abrogation properties of E79R and R82D HIV CA mutants on Trim5α and Trim5CypA.
Vero (AGM) (a) and Owl Monkey Cells (b). Cells were pre-treated for 3 hours with increasing amount of the indicated unlabelled virus before challenge with a fixed doses of wt HIV carrying an eGFP vector. The percentage of cells transduced was determined 48 hours later by flow cytometry. (c,d) Cyclophilin A incorporation of virions. Pelleted virions from wildtpye, E79R, R82D and G89V preparations were analysed by western blot for CypA (c) and CA (d).

Increasing amounts of virus, without genome, were added to Vero (African Green Monkey) cells or OMK (Owl Monkey Kidney) cells (to test abrogation of Trim5α or Trim5CypA restriction respectively), before challenge with a fixed amount of wildtype virus carrying an eGFP vector. The wildtype virus efficiently abrogates restriction in both cell lines, beginning to saturate the restriction factor with around 100μl added. Both mutants however were severely impaired in their ability to abrogate restriction, with up to 300μl of virus having a relative small effect upon restriction (Figure 6.11). These data are consistent with the proposition that these mutations may disrupt the formation or stability of the CA core, however it still remains a possibility that these mutations actually disrupt the restriction factor binding site, which may lie at the interface between hexamers.

In address this possibility, the level of CypA in virions was examined, reasoning that if the mutant virions could incorporate CypA normally then they should be equally capable of binding Trim5CypA (Figure 6.10c,d). Wildtype, E79R, R82D and the CypA binding site mutant G89V virions were examined by western blot of CypA. Particles were produced at equivalent amounts as measured by western blot for CA. Wildtype, E79R and R82D virions all incorporated cyclophilin A at similar levels. As reported previously, G89V incorporated significantly less CypA (Wiegers et al., 1999). These data suggest that the E79R and R82D mutations do not interfere with CypA binding and consequently, this is not the cause of the loss of ability to abrogate restriction in OMK cells.

6.5 Thin section EM analysis of viruses produced by E79R and R82D

Viruses were prepared for and examined by TEM (Figure 6.12). As previously observed, wildtype viruses show enormous heterogeneity in their internal structure (von Schwedler et al., 1998). Conical cores were often visible, occasionally with regions of greater electron density within them. Cores of rod shape, multiple cores and immature particles were also observed. Many E79R and R82D mutant particles also appeared to possess conical cores as well as the other forms. There was no clear difference between mutant and wild type preparations detected using this technique. For both wildtype and mutants, around 20-30% could be described as possessing condensed conical cores. This is consistent with previously published observations

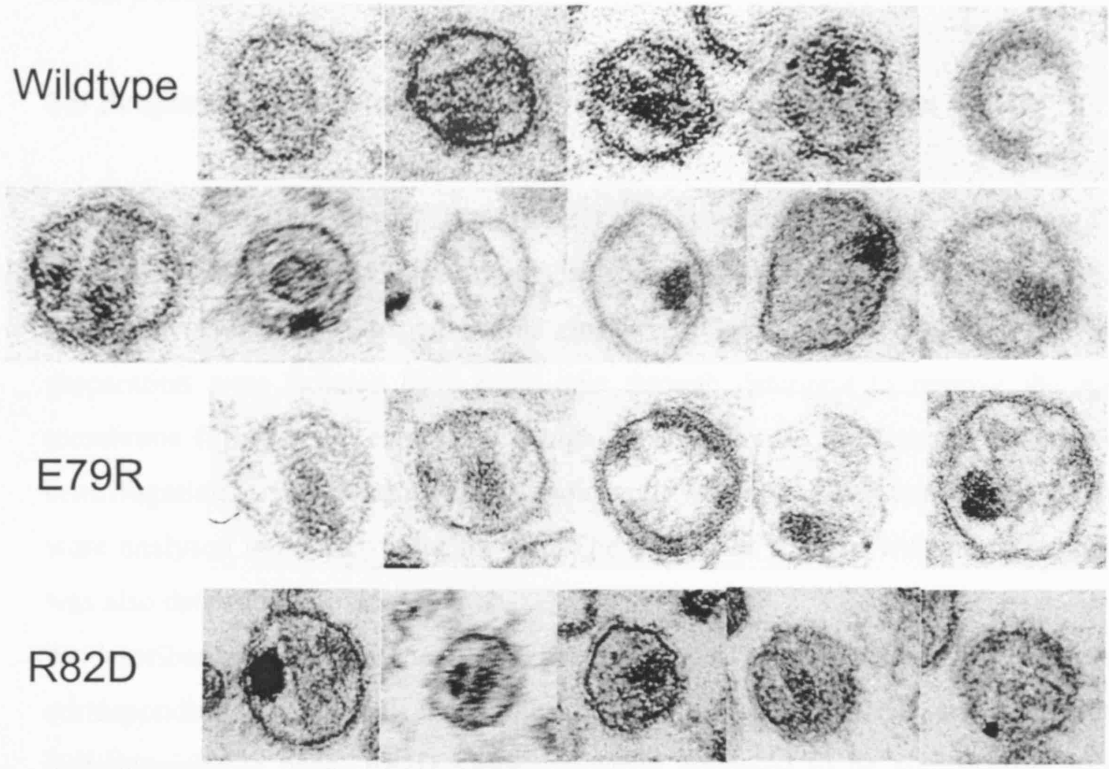


Figure 6.12 Thin section elctronmicrographs showing pelleted HIV particles from wildtype, E79R and R82D preparations.
Glutaraldehyde/Paraformaldehype fixed virions were pelleted by centrifugation and overlayed with agarose prior to staining with uranyl acetate as described in *Materials and Methods.* 50μM sections were analysed by electron microscopy.

using the same purification and imaging techniques (von Schwedler et al., 1998). The remaining 70% have been suggested to be made up of immature particles, and mature particles where the angle of section though the virus does not necessarily reveal a conical core.

6.6 Preparation and stability of E79R and R82D capsid cores

EM analysis revealed that E79R and R82D viruses were still capable of forming CA cores. In order to better characterise these cores, the detergent spin-through method described in detail in Chapter 5 was employed. Cores from a concentrated virus preparation were isolated by a rapid spin through detergent to remove the lipid membrane followed by capture in a high density sucrose gradient by equilibrium centrifugation. 1.5ml fractions from gradients of wildtype, E79R and R82D mutants were analysed by western blot for CA. The density of sucrose with these fractions was also determined (Figure 6.13).

As described earlier, wildtype viruses produced a peak of CA in a density of sucrose corresponding to around 1.22g/ml. E79R viruses produced an equivalent, indistinguishable peak. R82D viruses however appeared to be significantly more dense reaching more than 1.25g/ml, with more unprocessed and aberrantly processed material in association with the core. It is notable that in the particular experiment shown in figure 6.13, that despite the bottom fractions of the gradient being of slightly higher density (due to some variability during gradient pouring) in the R82D gradient, the virus still progresses further. The R82D viruses clearly therefore have a defect in the form of their CA cores which perhaps could explain the dramatic loss in infectivity observed.

It has been suggested that a core must be of optimum stability is necessary for successful infection. In order to test the stability and disassembly properties of the isolated cores, the disassembly assay described in detail in the previous chapter was employed.

Isolated cores were incubated with human cell extracts at 37°C for the times indicated and the percentage pelletable CA was determined at several time points. Using this system, the E79R mutant was found to disassemble around twice as fast as

the wildtype. Disassembly of the R82D mutant was not obviously affected in this assay (Figure 6.14).

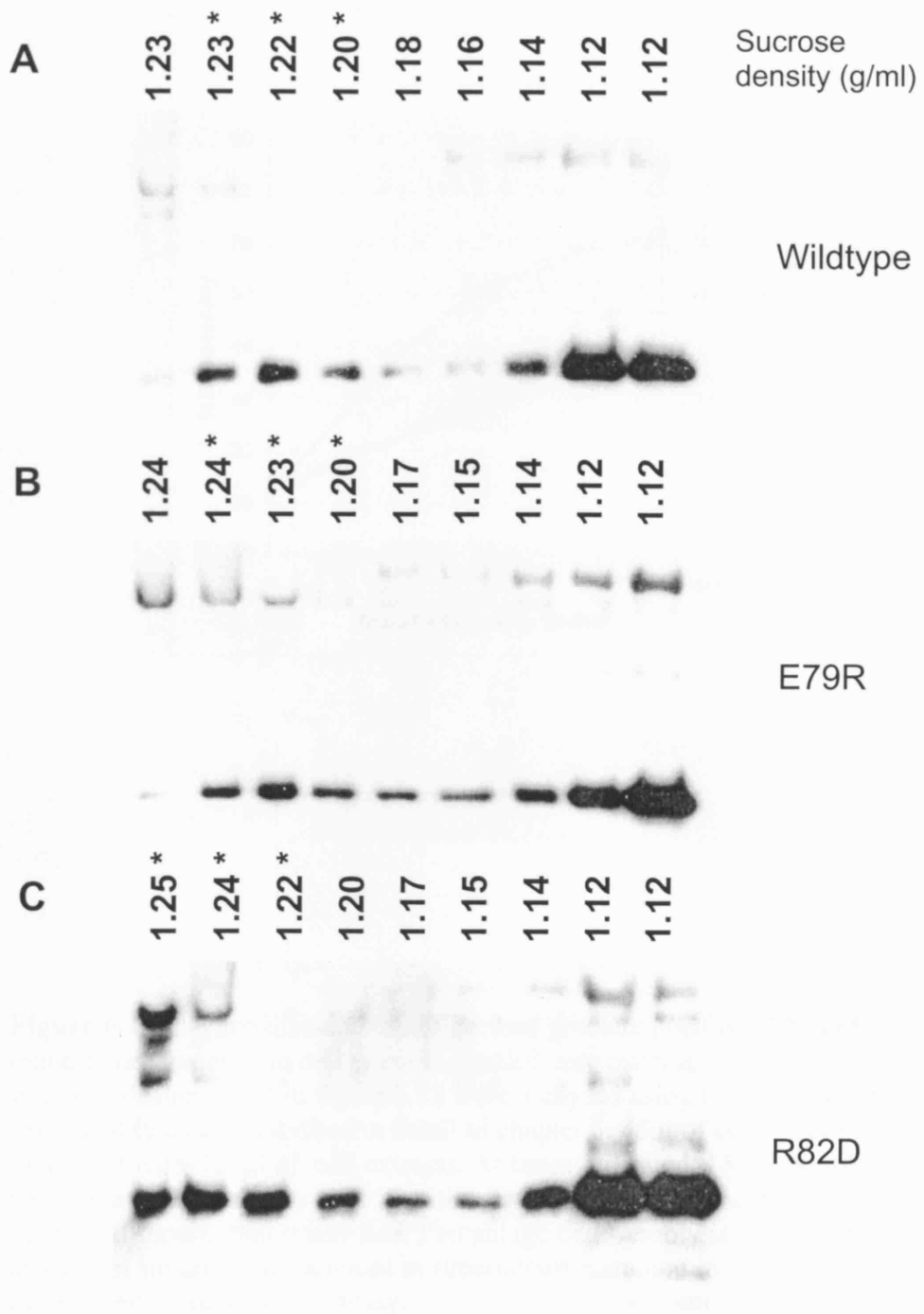


Figure 6.13 Isolation of Core Material from wildtype and mutant HIV particles. Core preparations were carried using the detergent spun through technique described in detail in Chapter 5. 250µl of a concentrated virus preparation were centrifuged through 1% Triton-X to remove lipid membranes. Cores were collected in a 30%-70% sucrose gradient. Sucrose gradients were harvested in 9 1.2 ml fractions. Samples from each fraction were subjected to SDS-PAGE and western blotting for CA. Sucrose density was determined from refractive index of the fraction. Fractions marked * were pooled, aliquoted, and frozen at -70°C for further analysis.

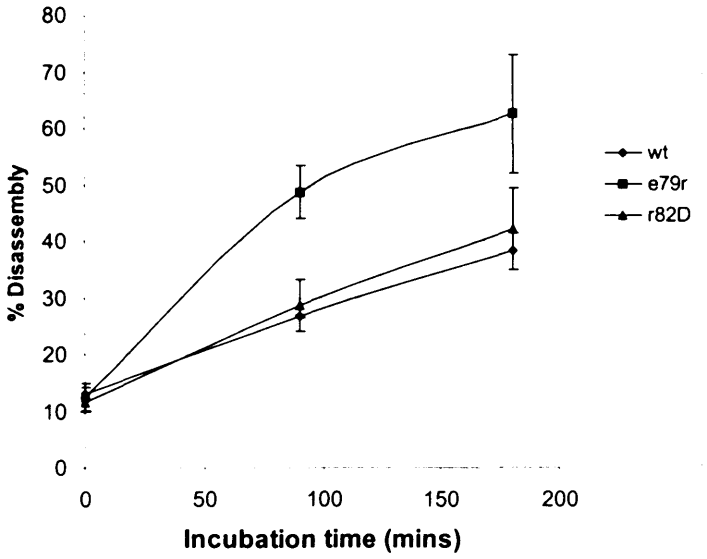


Figure 6.14 In-vitro disassembly of sucrose gradient purified HIV and mutant core material in cell extracts. Pooled core material from the fractions designated * in figure 6.13 were analysed using the cell extract disassembly assay described in detail in chapter 5. 45µl of cores were incubated with 415µl of cell extracts. At times indicated, 150µl samples were taken, separated by centrifugation at 100,000g and the amount in pellet and supernatant quantified. Percentage disassembly is derived from amount in supernatant/ (amount in supernatant + amount in pellet) as determined by p24 ELISA assay.

6.7 Discussion

This chapter has described experiments aimed at determining the biological importance of contacts observed within crystals of MLV capsid - a trimeric interaction involving D83 and R85. Mutations designed to disrupt this interaction by changing the polarity of the charge on these residues eliminated virus particle production, without affecting transport to the plasma membrane. Alanine substitution mutations at these positions did not significantly affect particle production indicating that D83 and R85 contacts are not essential for assembly. These mutations, however, did reduce the infectivity of the resulting virus. In contrast to many assembly mutants for both MLV and HIV-1, partial budding structures were rarely observed by electron microscopy in the case of D83R and never observed for R85D.

A recent study from the Singh group has also implicated the loop between helix 4 and 5 in MLV assembly (Auerbach et al., 2006). 11 amino acid insertions between positions G81 and D82 as well as P86 and T87 appear to block particle production and did lead to an accumulation of partial bud structures within the plasma membrane.

Clearly the method taken to disrupt contacts within this region can affect the phenotype observed. Arguably the most severe change is caused by altering the charge on either residue, not only blocking particle production but also any sign of assembly, although a direct comparison between these mutants and the Singh insertional mutants would be required to confirm this. It is conceivable that the substitution of the charge actually leads to repulsion between Gag molecules. The Singh insertions may not have such a drastic effect, blocking particle production but still allowing limited assembly. The alanine substitution mutants represent a mild disruption to the loop. Under these circumstances, other Gag-Gag contacts may be sufficient for assembly. The impact upon infectivity, however, suggests that either assembly is still in some way aberrant or that these residues are also important later in the lifecycle. Taken together, these data suggest that the D83 and R85 are closely positioned during virus assembly. Disruption of the charge on either of these residues is sufficient to completely abolish particle production.

The crystals used as the rationale for these experiments were composed of only the MLV CA NTD. The CTD is also very likely to make inter-hexamer contacts. In

addition, other regions of Gag undoubtedly have influence on the form of the hexameric lattice. It is also likely that the organisation of this lattice changes upon maturation. Despite extensive study, such a trimeric NTD interface has not been observed in 2D crystals of Gag/CA assembled on lipid membranes. For both HIV and MLV the NTD appears to form a dimeric interface between hexamers in those conditions most closely corresponding to immature Gag. (Huseby et al., 2005; Mayo et al., 2003). It is therefore interesting that the trimeric hexamer-hexamer contacts found in the NTD crystal impact so severely on MLV assembly when disrupted, although it is not possible to rule out dimeric contacts mediated by the same residues. Given the similar structures of HIV and MLV CA, it was important to determine whether contacts mediated by equivalently positioned residues in HIV CA are also important in virus assembly. In contrast to MLV, mutations designed to disrupt any potential E79 and R82 contacts by altering the polarity of the charge on these residues, did not affect particle production. This clearly indicates that these residues do not make critical contacts necessary for initial assembly and budding. This is consistent with published data showing that the entire N terminus of HIV CA can be deleted, whilst still allowing particle production (Accola et al., 2000). The viruses produced by the E79R and R82D mutations however are severely impaired in their infectivity, reduced to 2% and 1% respectively. Analysis by quantitative PCR indicates that these viruses are deficient in their ability to initiate reverse transcription after infection of the cell. When tested *in vitro* however, there was no effect on ability to initiate reverse transcription when compared to the wild type virus.

To better understand why disruption of CA might impact so severely upon reverse transcription and infectivity, a series of analyses were performed aimed at determining the consequence of disruption of these residues to the virus CA core.

Western blots revealed that CA is processed in both mutants potentially allowing core formation. TEM analysis revealed no obvious disruption to this process with cores visible in both mutants. When core material was isolated by detergent spin through into a sucrose gradient, a peak of wild type core material could be isolated at a sucrose density of 1.23-1.20 g/ml. The E79R mutant was unaffected in this. The R82D mutant however showed a different peak, with material detected at a density of 1.25g/ml and an accumulation of p55 Gag in these fractions, suggesting a defect in the composition of the R82D capsid core.

The proper stability and disassembly of the CA core has been linked to infectivity. In order to examine any effect on disassembly/stability in these mutants, material from the peak fractions for wildtype/E79R or the lowest density fractions for R82D was subjected to the disassembly assay described in Chapter 5. Surprisingly, the R82D mutant appeared to disassemble at a similar rate to the wildtype whilst the E79R mutant which possessed ostensibly similar cores disassembled significantly faster.

Finally, both mutants appeared to be defective in their ability to interact with restriction factors. This is consistent with an abnormal CA core structure (Forshey et al., 2005).

Both mutants clearly have dramatic effects on infectivity related to disruption of CA. R82D CA cores are more dense than wildtype perhaps suggesting improper maturation process. E79R cores are significantly less stable than wildtype, perhaps arguing that this may be the cause of the block to reverse transcription and the reduction in infectivity. These data therefore indicate that whilst not playing a major role in virus assembly, this region of HIV CA may play a role in the proper formation and stability of the mature virus capsid core.

Quite how this might be mediated is not completely clear. The organisation of CA and contacts made by the NTD are almost certainly different in the immature form of Gag when compared to the mature form (Mayo et al., 2003). Models suggest that the hexameric lattice in the mature form is less dense. The centre to centre distances between hexamers are greater in the mature form. This results in the NTDs being spaced further apart, perhaps prohibiting direct NTD/NTD contacts between hexamers. The primcipal hexamer-hexamer contacts are thought to be mediated by the dimeric CTD. (Briggs et al., 2006; Huseby et al., 2005; Li et al., 2000; Mayo et al., 2003).

Recent data which may explain this change in conformation derive from hydrogen-deuterium exchange experiments where it is suggested that upon processing and assembly into the mature hexameric lattice, contacts are made between the NTD and the CTD resulting in protection from exchange, due to a change in the orientation of the two domains with respect to each other. Specifically, the CTD which was previously thought to sit well below the NTD, separated by a flexible linker may in fact move up to bind the NTD and translate sufficiently in the XY plane to preclude NTD-NTD contacts when the CTD dimer is formed. The CTD may also make contacts with the NTD of subunits belonging to an adjacent hexamer (Lanman et al.,

2003; Lanman et al., 2004). This data supports a model where rather than two distinct layers within the lattice, the NTD and CTD are in fact in much closer Z-axis positions than previously thought.

E79R and R82D mutations produce severe phenotypes. Four possibilities present themselves which may explain this. i. The E79 and R82 mutations grossly disrupt the folding of the CA monomer. ii. This region is involved in binding a cellular factor necessary for proper core formation/disassembly. iii. The CA NTD and CTD domains move more freely than is currently thought and NTD-NTD contacts are possible. iv. This part of the NTD may interact with its own CTD or the CTD of an adjacent hexamer. The first two of these explanations seem unlikely as purified bacterially expressed E79R and R82D CA exhibit clear defects *in vitro* in both assembly assays and cryo-EM analysis of assembled tubes, despite circular dichroism spectra comparable to that of the wildtype protein (N. Mortuza, P. Rosenthal, personal communications). In addition, there is no indication *in vitro* that they are less stable, although the R82D mutant does show a very small proportion of aberrant processing products in virions. The third explanation does remain a possibility, however the available data consistently indicates that the NTDs are separated by too great a distance to make significant contact. The fourth possible explanation therefore of NTD/CTD contacts upon maturation mediated by a binding surface below these residues is compelling. Figure 6.15 depicts such an arrangement based upon that proposed by Prevlidge and colleagues. Full length CA monomers forming a dimeric inter-hexamer link via their CTDs are shown in green and orange. Nearby NTDs are shown in grey. E79 and R82 are marked in red. Figure 6.15, top, shows the dimeric interface from above the lattice, where E79 and R82 can be seen close to the CTD. Figure 6.15, bottom, shows a side view through the plane of the lattice. The CTDs are shown in the same plane as the NTDs, potentially available to contact E79 and R82. The arrangement shown here places the CTD in such a position that E79 and R82 may not make critical contacts (as indicated by the mild phenotype of alanine substitution mutations), but that a change of charge may cause sufficient repulsion as to destabilise or interfere with the formation of the lattice.

The model is shown for illustrative purposes only. One can conceive of several possible lattice organisations which would allow NTD/CTD contacts which differ from the one shown (Lanman et al., 2004). The orientation and location of the CTD in the mature lattice is still open to question. Recent unpublished data from full

length MLV CA crystals in a hexamer lattice currently being analysed suggest that the Z axis of the unit cell is too small to allow a CTD to sit below the NTD, supporting the above model (I. Taylor, personal communication).

The data presented here show that interference with E79 and R82 in HIV-1 CA can have a dramatic effects upon the virus. Single amino acid changes within this region can essentially abolish the infectivity of HIV. Such effects could potentially be explained by NTD/CTD interactions. This region therefore represents a potential therapeutic target in the treatment of HIV infection.

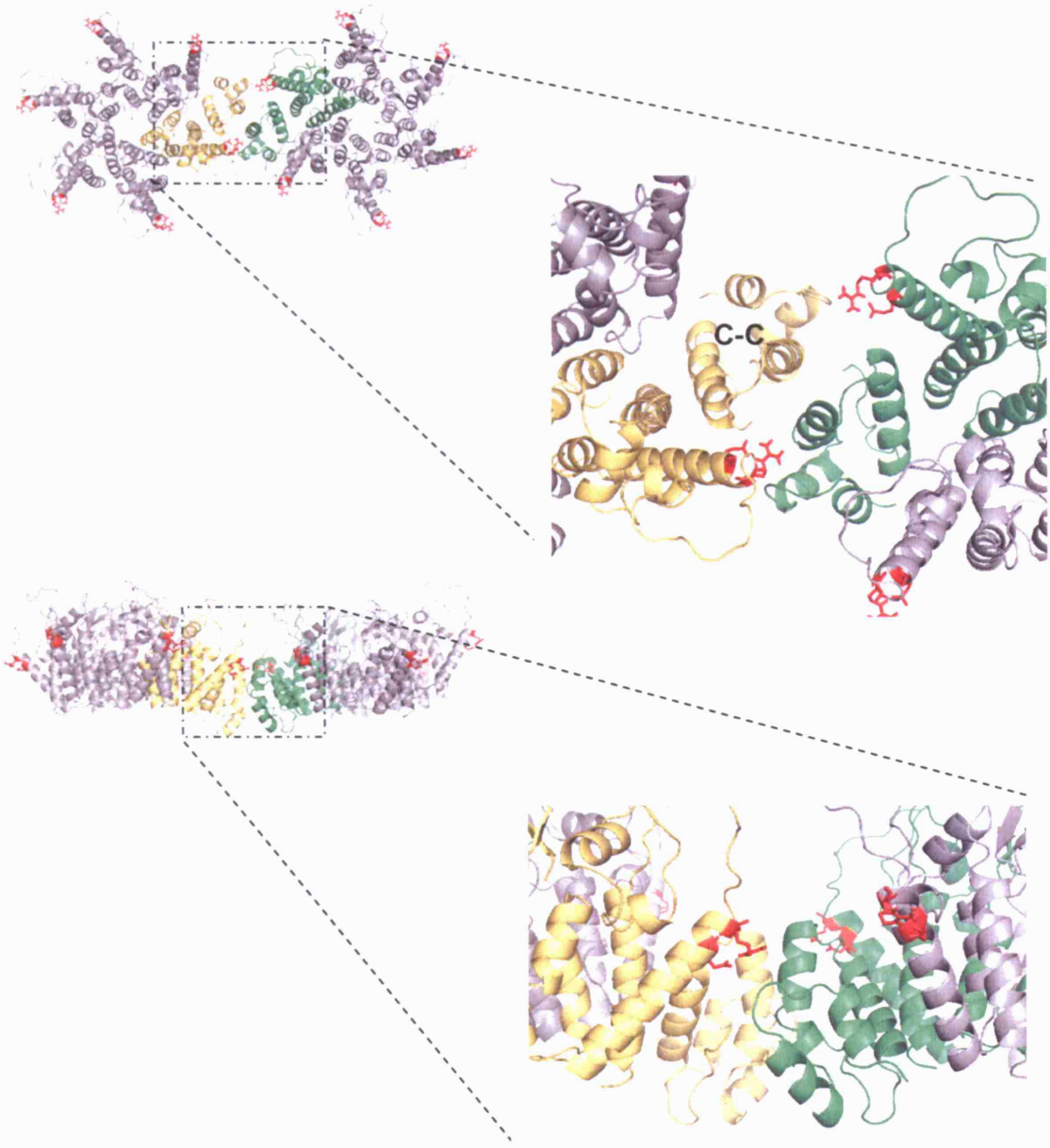


Figure 6.15 Model showing association of HIV CA NTD and CTD. (Top) Two hexamers of NTDs are shown associated by dimeric CTD interactions (only 2 CTDs are shown for clarity). Each full length monomer is shown in colour. On the NTDs, E79 and R82 are shown in red. (Bottom) Side view showing CTDs in plane with NTDs.

CHAPTER 7

Investigations into the role of Trim5α localisation and cell biology in restriction

In investigating the interactions between restriction factors and retroviral capsid, the cell biology of the restriction factor cannot be ignored. The virus must encounter the restriction factor at some point after fusion and release of the capsid core into the cytoplasm. The fate of the virus between this point and nuclear import is not well defined. Studying the localisation of restriction factors may therefore shed light on this important phase of the lifecycle as well as giving insight into the mechanism of action of the restriction factors themselves.

The importance of factor localisation in restriction is unknown. Although both endogenous and over expressed Fv1 have been found in association with the trans-golgi network it has not been confirmed that this is the subset responsible for restriction, a processing intermediate or perhaps simply a non-functional accumulation. An undetectable, low level of diffuse cytoplasmic protein has been suggested as a functional alternative. However, Int1, a Fv1 internal delection mutant which mislocalised to the endoplasmic retriculum fails to restrict MLV and exerts a dominant negative effect over endogenous Fv1 (Yap and Stoye, 2003).

Tripartite motif (TRIM) family of proteins have been suggested to occupy a variety of cellular compartments. Trim19 (also known as PML) for example localises to nuclear bodies; Trim1 appears to be associated in filamentous structures with the microtubule network whereas Trim28 associates with specific chromatin domains within the nucleus. Prior to its identification as a restriction factor, over expressed Trim5α, like several other Trims had been shown to form speckles within the cytoplasm, often described as cytoplasmic bodies, which are not apparently associated with any known cellular compartment. Common to all characterised Trim family members is an ability to self-associate (Reymond et al., 2001).

Trim5α can be labelled on either its N or C terminus with a variety of fluorophores or epitope tags without affecting its antiviral activity (Perez-Caballero et al., 2005b; Song et al., 2005a; Stremlau et al., 2004). Regardless of the tag used, Trim5α still

forms cytoplasmic bodies. GFP-Trim5α therefore represents a useful tool to begin examining the cell biology and localisation of this restriction factor.
This chapter describes experiments designed to describe the localisation of Trim5α and characterise its importance.

7.1 Localisation of GFP-Trim5α

Live TE671 cells stably expressing GFP-tagged human or rhesus macaque Trim5α from a retroviral vector were examined by fluorescence deconvolution microscopy (Figure 7.1). The observed GFP signals were indistinguishable. Both showed fluorescent puncta distributed throughout the cytoplasm surrounded by a diffuse cytoplasmic signal. No fluorescent material was observed within the nucleus. These observations confirmed data existing at the time and have been subsequently published by a number of groups (Javanbakht et al., 2005; Perez-Caballero et al., 2005a; Song et al., 2005a).

7.2 Trim5α cytoplasmic bodies are highly motile

Immediately upon examination of live cells expressing GFP-Trim5α, it became clear that cytoplasmic bodies within these cells were highly motile, with rapid movement visible simply by observing live cells through the microscope objective in real time. In order to record this movement, images were rapidly captured in sequence at the shortest possible time interval compatible with obtaining a sufficiently long exposure. Supplementary movie 1 shows such a 60 frame sequence captured at 1.325 second intervals. The high degree of motility is clear. There appear to be two types of movement. Several of the bodies show multidirectional short saltatory movements, whereas others show rapid uni-directional long distance movements.
The representation of such complex data captured in digital image sequences in a printed format is challenging however figure 7.2 shows one method. Using an image addition process, the 60 frames from supplementary video 1 were combined into one image on the basis that in the composite image, a given pixel is assigned the maximum intensity value observed throughout any of the 60 input images (Figure 2,

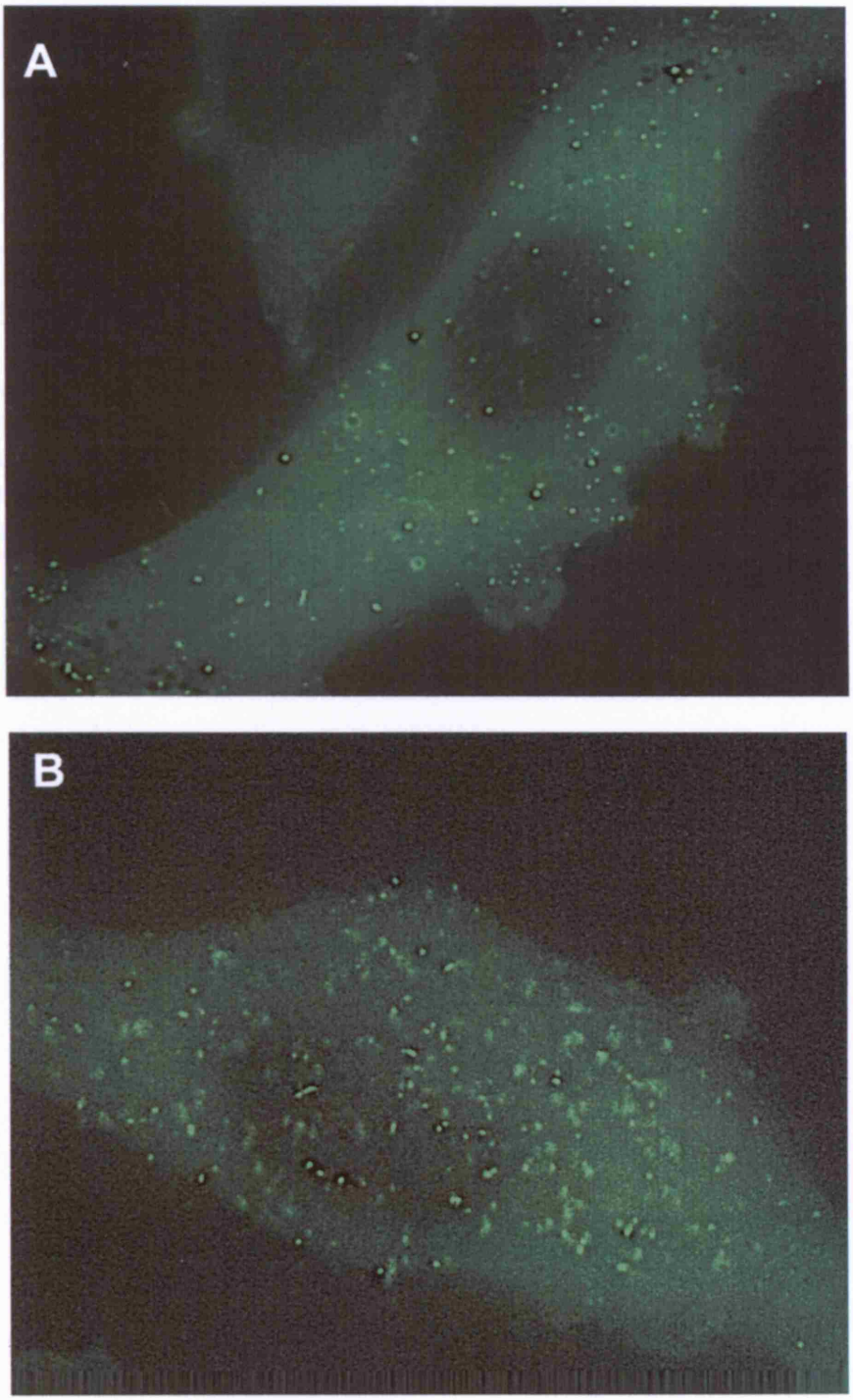
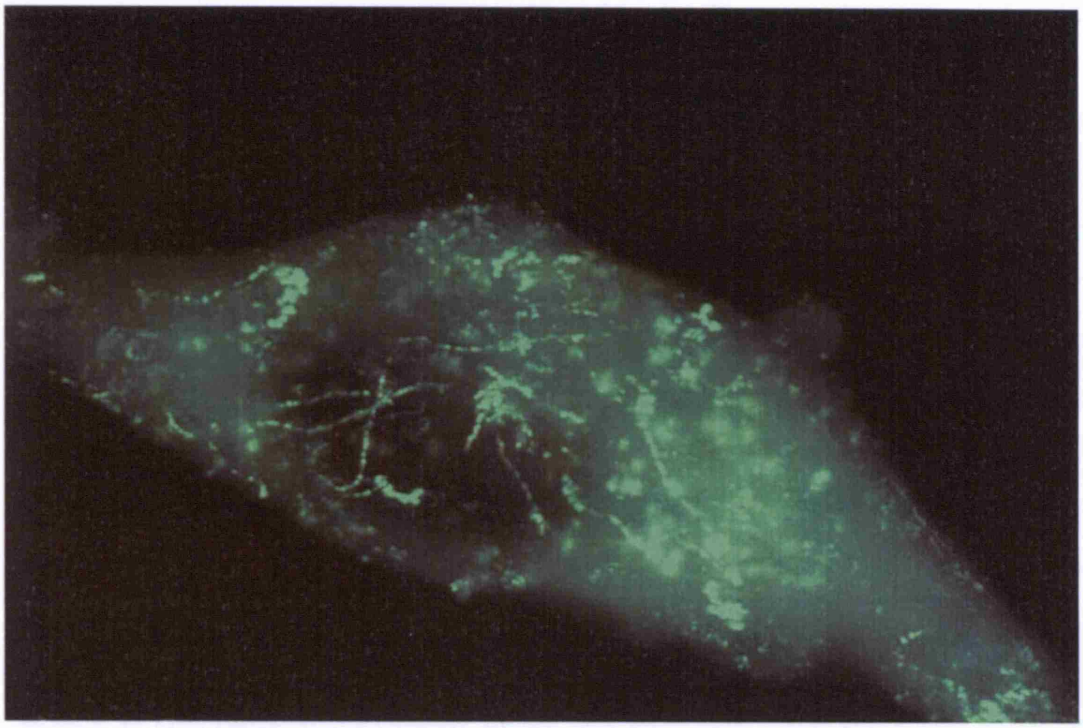
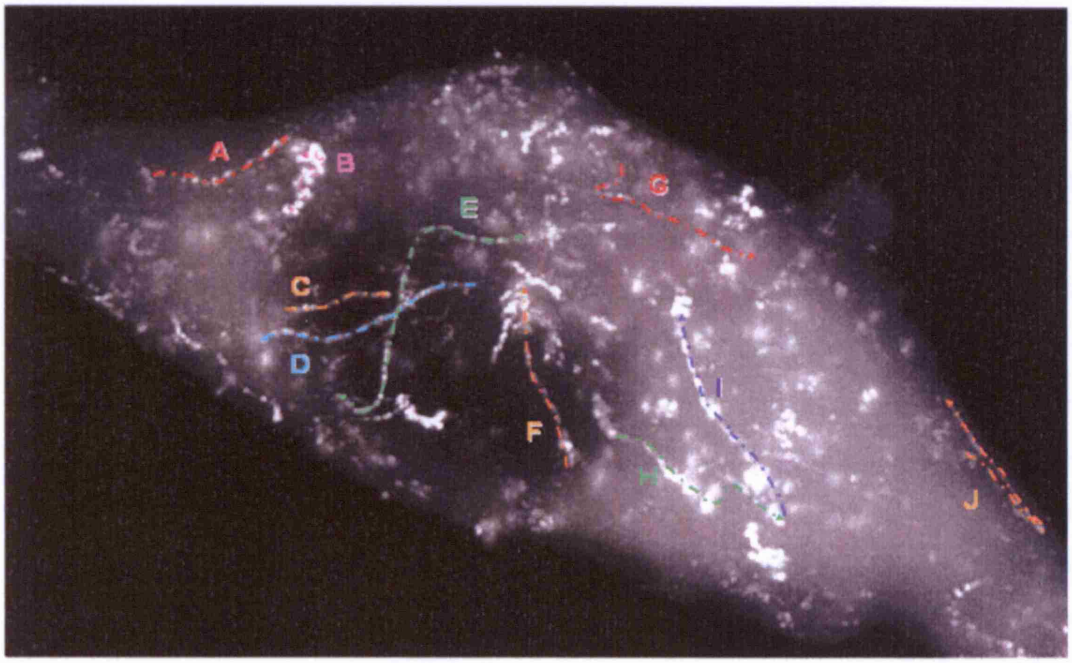


Figure 7.1 Fluorescence images showing cytoplasmic localisation of GFP-Trim5α of rhesus macaque (Rh) (a) and human (Hu) (b) origin.
Cells were transduced with retroviral expression vectors expressing either GFP-RhTrim5α or GFP-HuTrim5α at a multiplicity of infection of approximately 10. After 2 weeks of culture, live cells were analysed by fluorescence de-convolution microscopy.

Figure 7.2 Composite image showing particle movement from the image series in supplementary movie 1.
A live TE671 cell stably expressing GFP-HuTrim5α was imaged over 60 frames at 1.325 second intervals (0.75 frames per second). The 60 individual frames were summed using Image J on the basis that in the composite image, a given pixel is assigned the maximum greyscale value (0-256) observed in any of the 60 frames throughout the series (Top). This process clearly shows the tracks of a number of cytoplasmic bodies. The directionality and authenticity of these tracks were confirmed by comparison with movie 1. Several prominent tracks were identified for further analysis. These are labelled A-J (Bottom).




A
B
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Top). This process clearly shows a number of tracks. These can be confirmed and the direction of

movement determined by comparison with video 1. Several such tracks and their direction are identified and labelled A-J (Figure 2, Bottom).

The short distance saltatory movements are exemplified by particle B, whereas the others the others show longer distance uni-directional movement.

In order to better analyse and represent the movements of these particles, the inter-frame movement rates of the indicated bodies were measured using the manual tracking function of the particle tracking software GMview (G. Mashanov, NIMR). This software allows the position of the particle to be recorded between each frame and as the time interval is fixed, the velocity can be easily determined. The data from such an analysis is shown in figure 7.3. The horizontal z-axis shows the time index in the image sequence in during which the particle was observed. The vertical y-axis shows its step (inter-frame) speed. Using this approach, a particle's fate throughout the entire sequence can displayed.

Particle A shows periods of rapid movement followed by apparent stagnation. Over the time it was observed, the average speed of particle A was 0.1μm/sec, however at least 3 burst of rapid directional movement were observed of 0.4μm/sec, 1.48μm/sec and 0.3μm/sec. These rapid movements can also be observed in supplementary movie 1. Particle B shows short, low speed movements throughout most of the time it is observed however a number of more rapid movement bursts are observed towards the end of the movie up to 0.8μm/sec. Other particles such as C, G and J show very rapid although still variable movements through out their observation time, with maximum speeds up to 2.3μm/sec observed. Particle J for example maintains a high average speed of 1.6μm/second. Due to their very high motility, these particles could only be tracked for a short time before they left the focal plane. There was not an obvious preference for movement either to or from the cell periphery. Particles apparently moving in either direction were readily observed. Particle J in fact appears to change direction during the time it was tracked, although not on exactly the same path. It should also be noted that many more rapidly moving particles may exist but are not observed due to the limits of the detection technology (frame rate and camera sensitivity) used here.

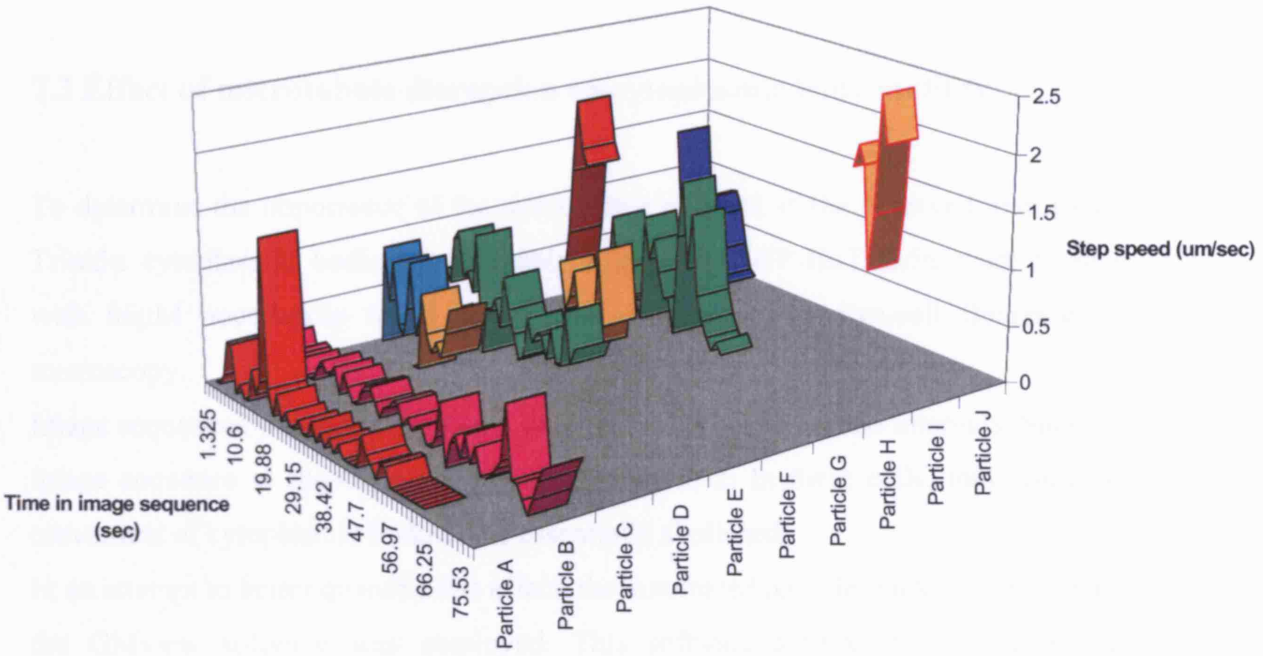

Figure 7.3 Chart showing movement rates for a selection of cytoplasmic bodies. The velocities of the particles A-J in figure 7.2 were measured thoughout the time that they were observed during the 60 frame (80sec) sequence using the manual tracking function of the particle tracking software GMview (see materials and methods). The vertical y-axis shows the average speed of the particle between each consecutive frame (step speed, μm/sec), the horizontal-z axis shows the relative time during the image sequence in which the particular particle was observed.

Such rapid, long distance directional movements of above 1μm/sec suggest an active mechanism of movement and strongly implicate the microtubule network as the carrier.

7.3 Effect of microtubule disruption on cytoplasmic body motility

To determine the importance of the microtubule network in the observed motility of Trim5α cytoplasmic bodies, cells stably expressing GFP-HuTrim5α were treated with 66μM nocodazole for 2 hours prior to analysis by live-cell fluorescence microscopy.

Image sequences were again captured in sets of 60 at 1.325 second intervals. Such an image sequence is shown in supplementary movie 2. In these cells, long distance movement of cytoplasmic bodies was essentially abolished.

In an attempt to better quantify this effect, the automated particle tracking function of the GMview software was employed. This software automatically follows the movement of point sources of fluorescence (such as Trim5α cytoplasmic bodies) within an image. A particle will be tracked if above a given size and intensity. Tracks are made between nearest neighbours in consecutive frames. A minimum length of track (number of frames) can be specified as can the maximum movement allowed between frames (number of pixels). Each track can be manually verified as authentic before further analysis.

Such an analysis has advantages and limitations. The primcipal advantages are that a huge amount of data can be analysed relatively quickly and efficiently and that data sets can be compared without any unintentional bias on the part of the experimenter.

This is balanced by the disadvantage is that the fastest moving particles are not tracked efficiently because rapid movement often means that nearest neighbours in consecutive frames are not derived from the same particle. This results in output skewed towards lower velocities. Nonetheless, the ability to examine objectively a large number of samples is ideal for comparing nocodazole treated and untreated cells.

Figure 7.4 shows data from such an analysis. Image sequences of 60 frames were recorded for 5 cells in nocodazole treated and untreated cells. This resulted in 258 tracks for untreated cells and 306 tracks for nocodazole treated cells. Each track was

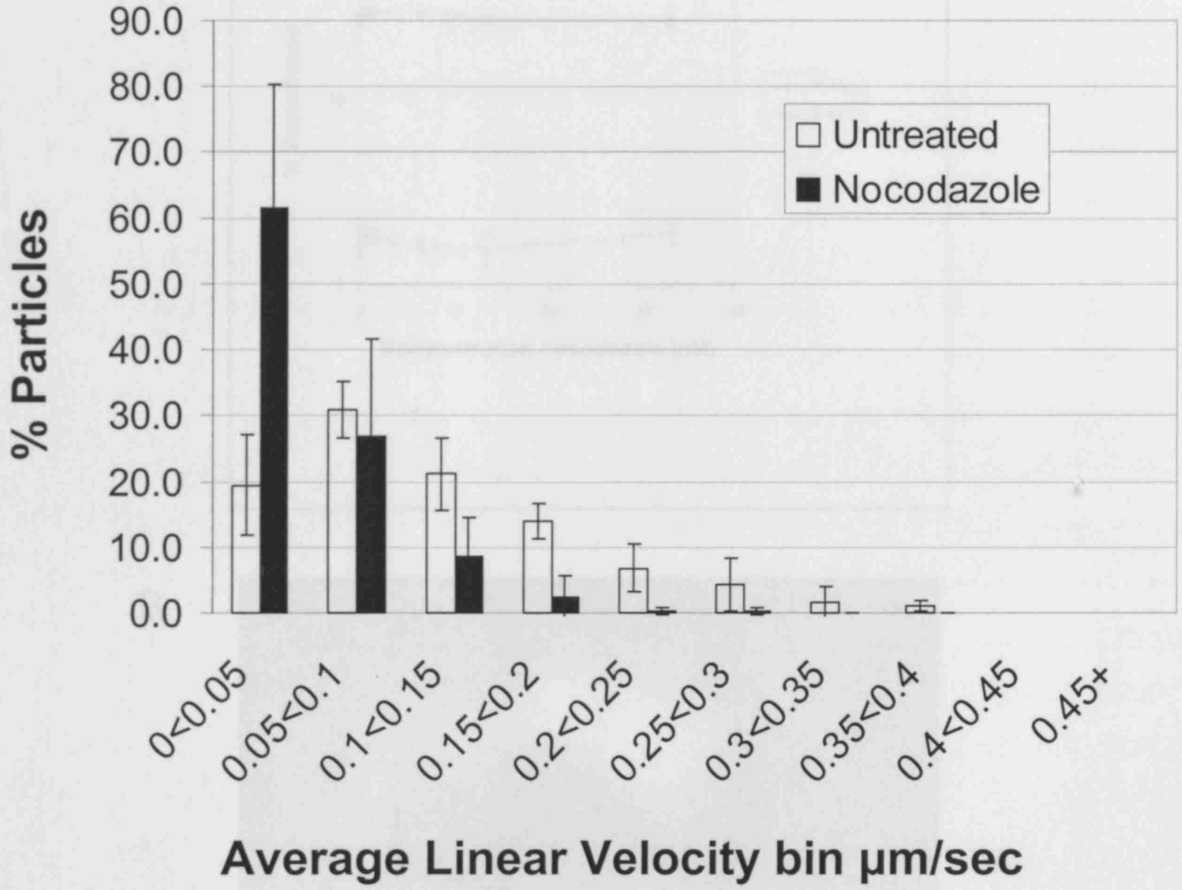

Figure 7.4 Reduction in cytoplasmic body motility upon treatment with nocodazole.

The movement of GFPTrim5α in TE671 cells stably expressing GFP-Trim5α was monitored by live-cell microscopy. Cells were either left untreated or treated with 66µM nocodazole for 2 hours prior to imaging. Images were captured at a rate of 0.75 frames per second for a total of 60 frames. Particle tracks were analysed using GMview as described in *Materials and Methods*. This software was used to calculate the average linear velocity of each particle over the time that it could be unambiguously tracked. Each particle track was verified manually in order to confirm its authenticity.
5 randomly chosen cells were analysed in each case resulting in 258 cytoplasmic body tracks for untreated cells and 306 tracks for nocodazole treated cells. Velocities were grouped into 0.05µm/second bins for each cell analysed and the percentage particles in each bin determined. The mean and standard deviation values in each bin over 5 cells were then calculated.

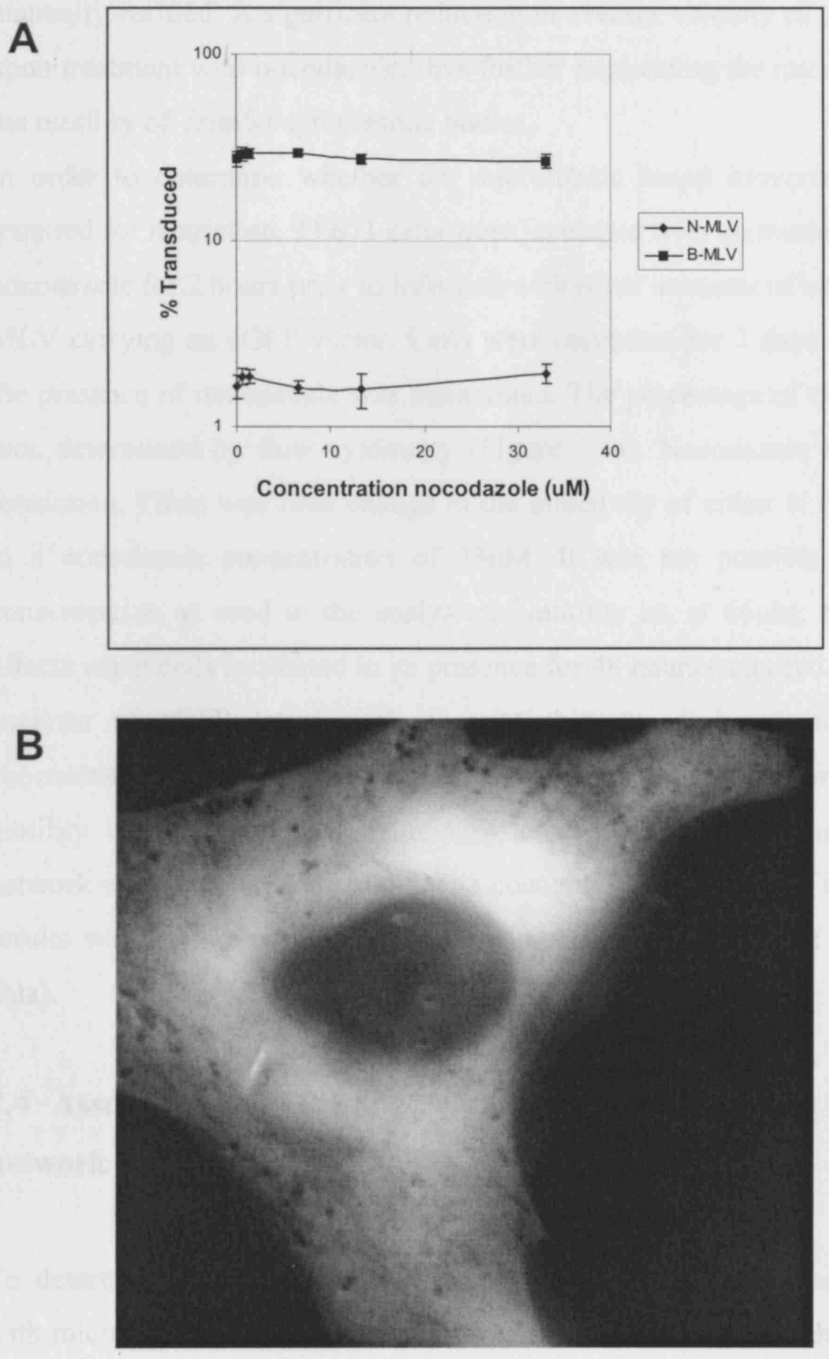

Figure 7.5 Effect of treatment with nocodazole on restriction by Trim5α in TE671 cells.
(a) Cells were treated with the indicated concentration of nocodazole for 2 hours prior to infection with a fixed amount of N or B-MLV (of equal titre on *M.dunni* cells). The presence of nocodazole was maintained. After 2 days, the percentage of cells transduced was determined by flow cytometry. (b) Cells expressing CFP-α-tubulin were treated with 33μM nocodazole for 2 hours prior to analysis of live cells by microscopy.

manually verified. A significant reduction in average velocity of particles is apparent upon treatment with nocodazole, thus further implicating the microtubule network in the motility of Trim5α cytoplasmic bodies.

In order to determine whether the microtubule based movement was absolutely required for restriction, TE671 cells were incubated with increasing concentrations of nocodazole for 2 hours prior to infection with equal amounts of either N or B tropic MLV carrying an eGFP vector. Cells were incubated for 2 days during which time, the presence of nocodazole was maintained. The percentage of cells transduced was then determined by flow cytometry (Figure 7.5a). Nocodazole had no effect upon restriction. There was little change in the infectivity of either N or B tropic virus up to a nocodazole concentration of 33μM. It was not possible to test the 66μM concentration as used in the analysis of motility as, at 66μM, the drug had lethal effects when cells incubated in its presence for 48 hours (required for flow cytometry analysis of eGFP expression). Despite this, the lack of any effect up to a concentration of 33μM argues against an absolute requirement for microtubule based motility in the activity of Trim5α. Microscopy confirmed that the microtubule network was completely disrupted at a concentration of 33μM (Figure 7.5b). Similar results were obtained in separate experiments using HIV-1 (M. Yap, unpublished data).

7.4 Association of Trim5α cytoplasmic bodies with the microtubule network

To determine whether Trim5α cytoplasmic bodies can be observed in association with microtubules, cyan fluorescent protein (CFP) was fused to the N-terminus of α-tubulin and sub-cloned into a retroviral vector. Stable cells were then derived by transducing TE671 cells with this construct as well as GFP-HuTrim5α. Live-cells expressing both fusion proteins were observed using a fluorescence microscope. Figure 7.6 shows such a cell. Cytoplasmic bodies can clearly be seen in association with the microtubule network.

If images are captured in sequence, movement on microtubules is apparent (see supplementary video 3) although this is hindered by the requirement to capture

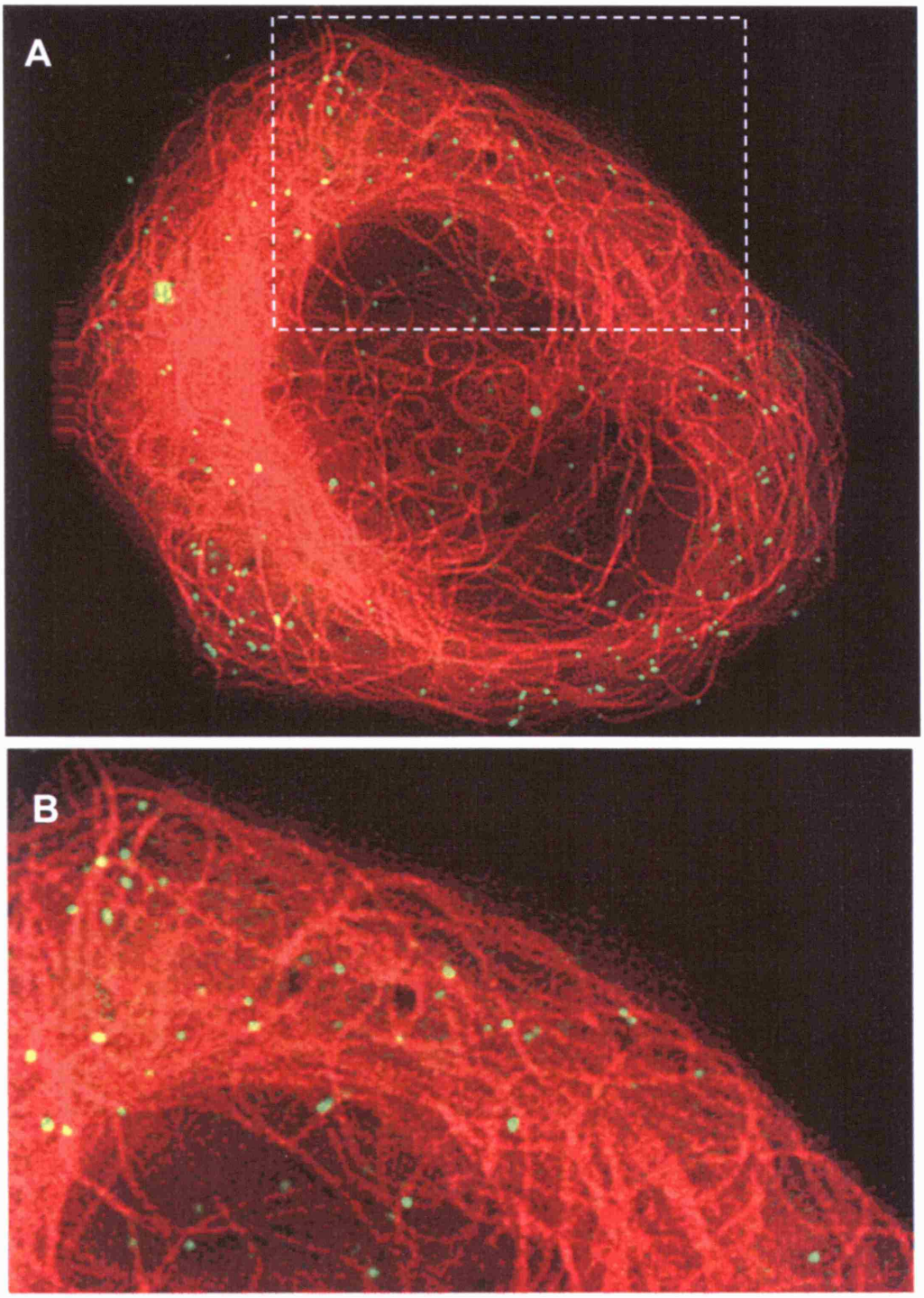

Figure 7.6 Live-cell fluorescence images showing GFP-Trim5α cytoplasmic bodies in association with the microtubule network.
TE671 cells stably transduced with CFP-tubulin and GFP-Trim5α retroviral expression vectors were observed using a fluorescence microscope. CFP is show in red for ease of viewing. GFP is coloured green. (A) Whole cell (B) Highlighted area.

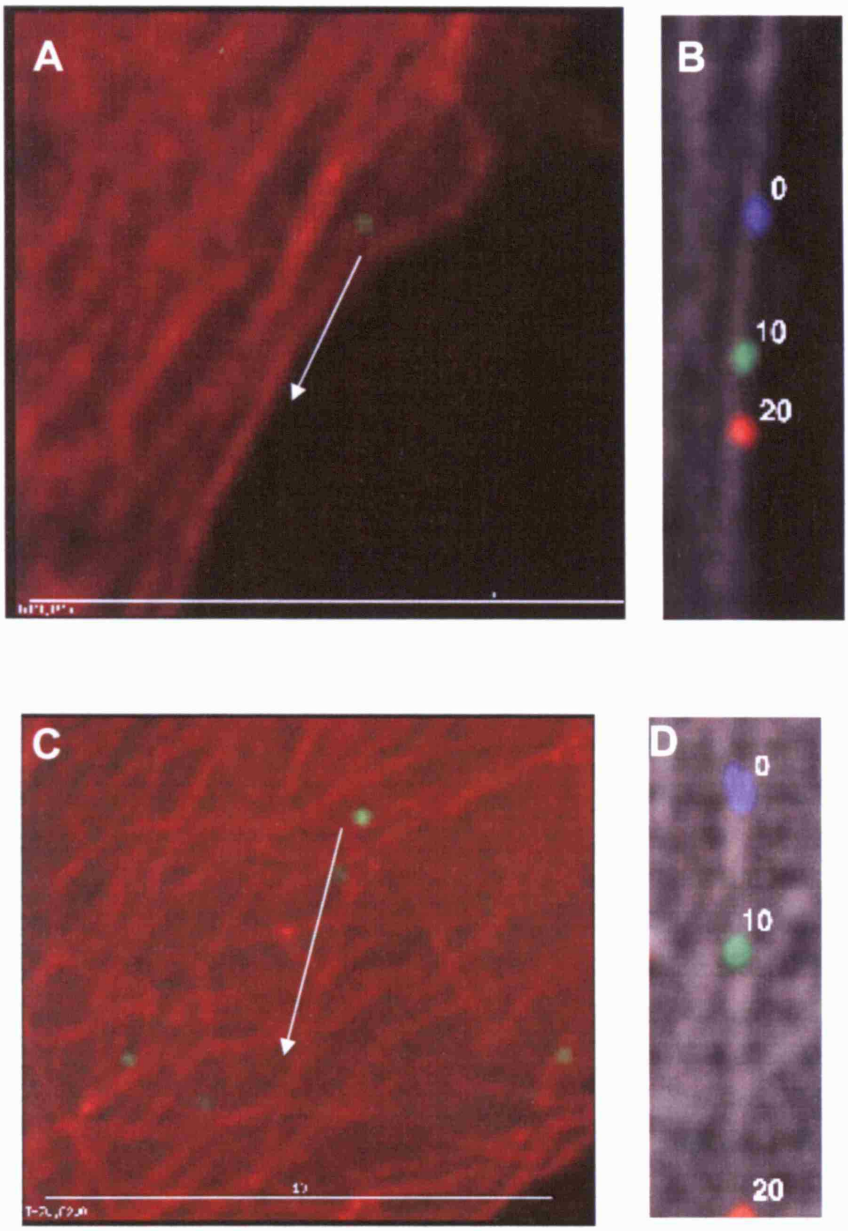

Figure 7.7 Movement of individual cytoplasmic bodies upon microtubules. Panels A and C show starting positions for two cytoplasmic bodies (green) and the microtubule network (red). The direction of movement is indicated. Panels B and D show the microtubule network in grey with the relative positions of the cytoplasmic body at 10 second intervals coloured blue, green and red. The relative time (sec) is noted by the body.

images for both CFP and GFP resulting in an increase in the frame rate to 10 seconds.

Figure 7.7 shows movement of 2 cytoplasmic bodies on microtubules. To represent this movement clearly in print, the microtubule network and the first image in the sequence is shown in A and C, with the direction of movement indicated. B and D show the microtubule network in grey with the GFP-signal from 3 successive frames shown in blue, green and red respectively.

7.5 Effect of the HSP90 inhibitor geldanamycin on Trim5α cytoplasmic bodies

During the course of these studies, a report from Sodroski and colleagues suggested that upon treatment with the HSP90 inhibitor geldanamycin (GA), GFP-Trim5α cytoplasmic bodies were disrupted (Song et al., 2005a). The suggestion was made that the material contained within the cytoplasmic bodies became diffusely distributed throughout the cell. Under these conditions, HIV-1 was still efficiently restricted. The authors therefore concluded that Trim5α cytoplasmic bodies were not required for restriction and further surmised that Trim5α cytoplasmic bodies form as a consequence of over-expression and are in fact protein aggregates awaiting destruction by the cellular proteolytic machinery. In support of this hypothesis, HSP90 has been shown in association with aggresomes and some larger Trim5α cytoplasmic bodies.

HSP90 however is primarily a key cellular chaperone protein involved in the folding, stability and transport of a huge range of client proteins. Given that its inhibition clearly had a dramatic effect on Trim5α localisation, if not its activity, it was felt important to further study its effects.

In order to confirm the published data regarding the effect on GFP-Trim5α localisation, TE671 cells stably expressing GFP-HuTrim5α were treated with 10μM GA for two hours prior to live-cell microscopy (Figure 7.8). Panel A shows an untreated cell, panel B shows a cell treated with geldanamycin imaged and processed under identical conditions. Cytoplasmic bodies appear to be absent although an aggregate of fluorescent material is apparent. There is essentially no diffuse signal. If the image in B is digitally enhanced by adjusting its brightness and contrast, it

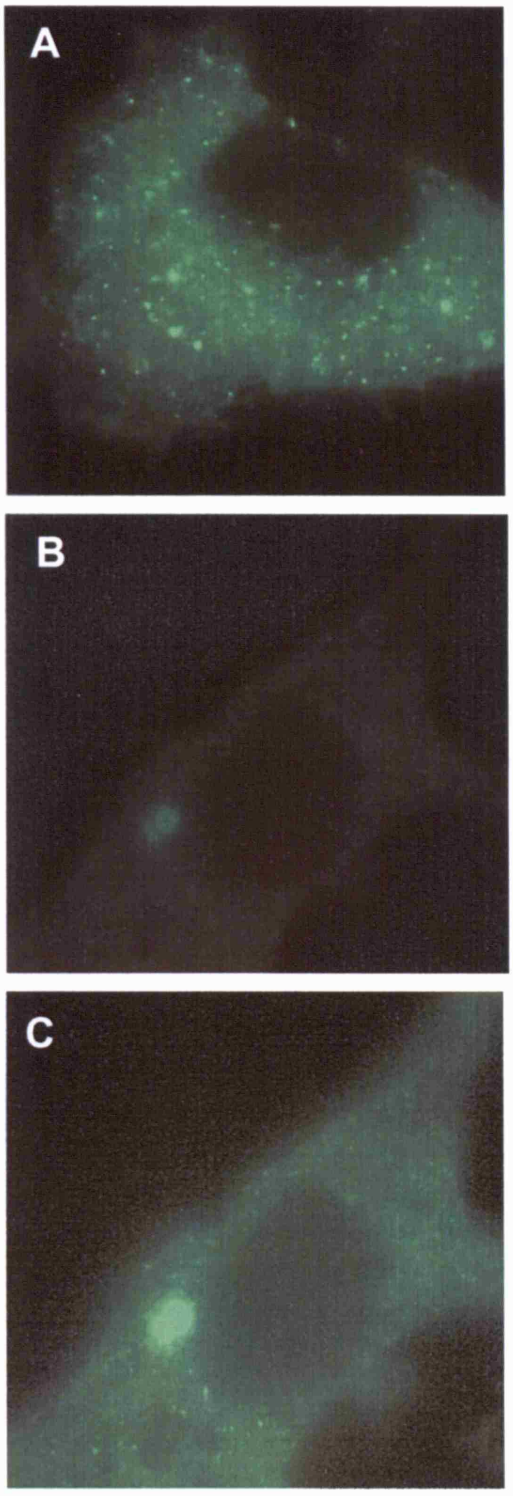

Figure 7.8 Effect of geldanamycin treatment on GFP-Trim5α localisation. TE671 cells stably transduced with GFP-Trim5α were treated with 10μM geldanamycin for 2 hours prior to microscopy. (a) Untreated cells, (b) Geldanamycin treated image processed in an identical manner to A. (c) Contrast enhanced image of B.

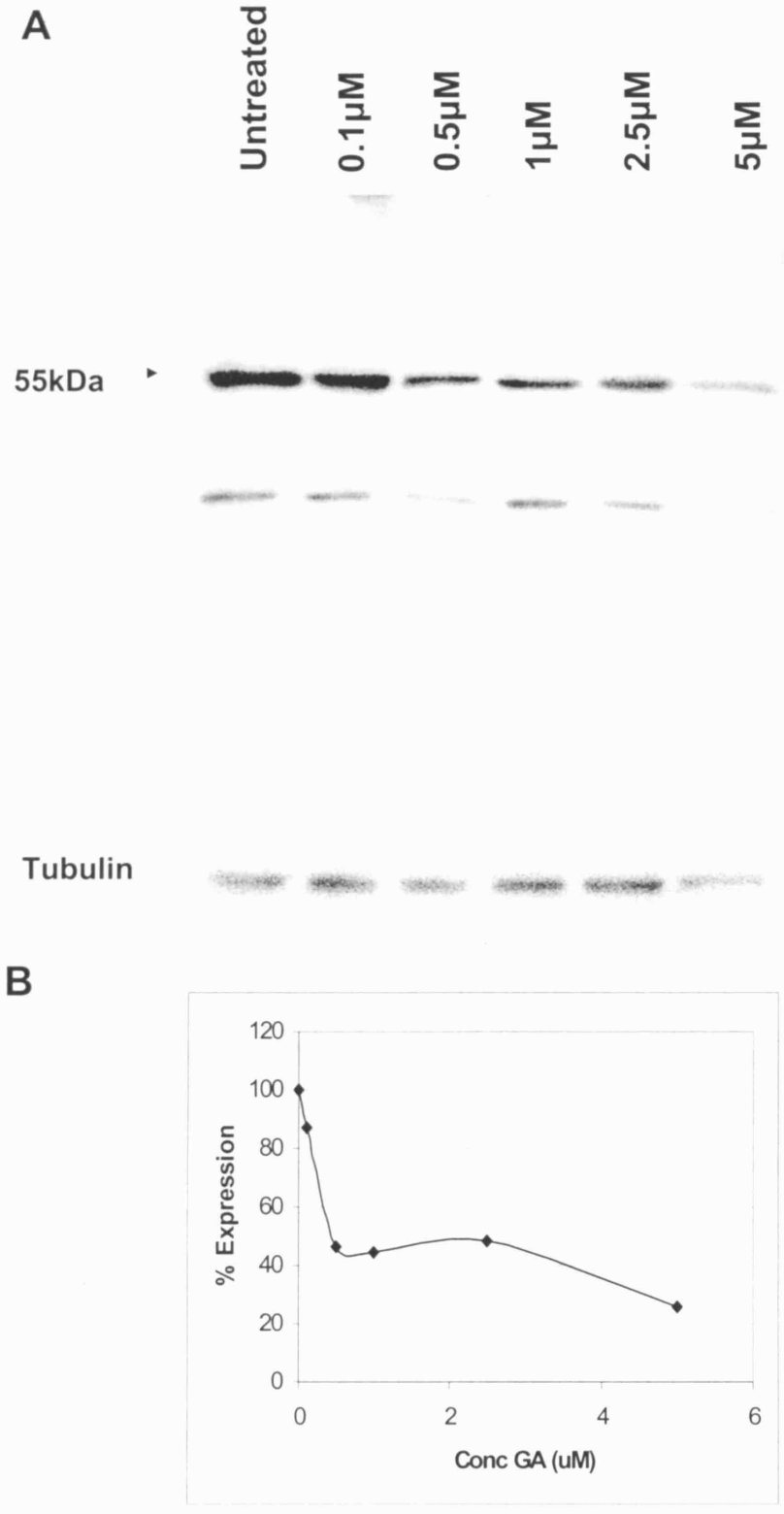

Figure 7.9 Effect of geldanamycin treatment on Trim5α expressing TE671 cells. Cells were treated with the indicated concentration of geldanamycin for 2 hours prior to lysis and analysis by western blot (a) Trim5 was detected using a polyclonal antibody directed against the N-terminus of the protein. (b) The intensity of the bands due to Trim5α was quantified and the relative expression compared to untreated cells determined.

becomes possible to observe much fainter structures (Panel C). Clearly a similar number of cytoplasmic bodies are still present but are much less intense. The aggregate of fluorescent material is shown to be a peri-nuclear accumulation. This was found in every cell examined.

The data here contradict the published data, which suggested that there was an increase in diffuse cytoplasmic signal and made no mention of the accumulation of a peri-nuclear aggregate.

7.6 Reduction of Trim5α levels by treatment with geldanamycin

Upon treatment with GA, cytoplasmic bodies are still present although less intense. There is virtually no detectable diffuse signal. It is difficult to reconcile these data with the hypothesis that HSP90 inhibition causes dissolution of Trim5α cytoplasmic bodies. It seemed possible that a reduction in protein levels could be responsible. In order to test this hypothesis, TE671 cells stably expressing HuTrim5α from retroviral vectors were treated with increasing concentrations of GA for 2 hours. Cells were lysed and the lysates analysed by western blot, probing for Trim5α using a polyclonal antibody directed against the N-terminus of the protein (Figure 7.9a). GA treatment caused a reduction in the expression level of Trim5α. Around a 75% reduction was observed after treatment with 5μM GA.

Although expression of Trim5α reduced, there is clearly still Trim5α present. It seemed possible that this could explain why restriction is apparently unaffected by GA treatment. In the original study, and in these experiments, Trim5 is over expressed. It therefore seemed important to examine the effects of GA in wild type cells and to clarify any differences in expressions levels.

7.7 Relative expression of Trim5α in a panel of cell lines

In order to examine the relative levels of Trim5α expressed in cells transduced with retroviral expression vectors to those in wild type cells, expression levels in a panel of cell lines were examined by western blot.

M. dunni cells do not express Trim5α and so serve as a negative control. Expression of a siRNA in human TE671 cells has been shown to inhibit restriction and so also

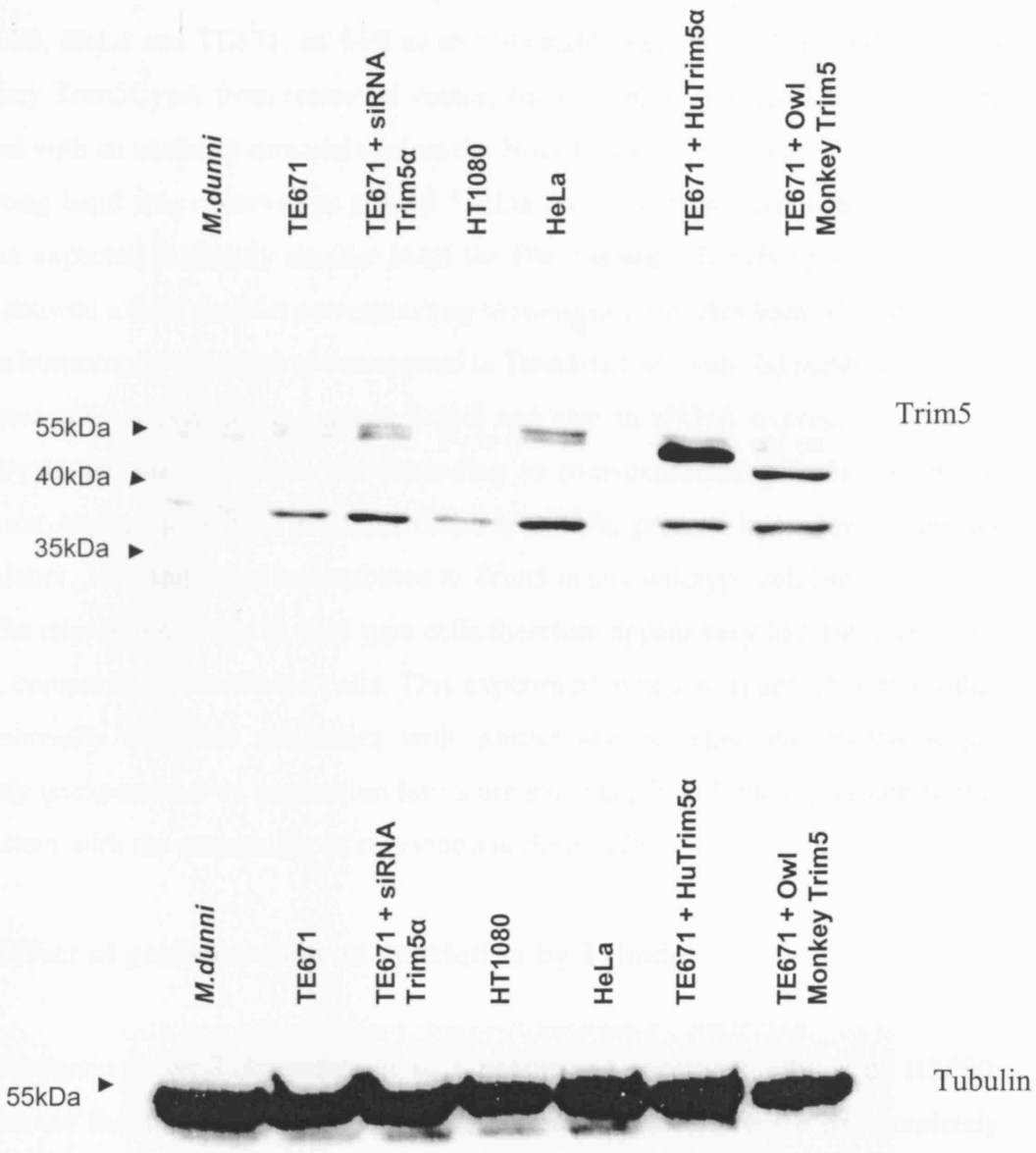

Figure 7.10 Western blot probing for Trim5α in a panel of cell lines.
The indicated cell types were grown to confluency in a 6cm dish then lysed in 500µl of triple detergent lysis buffer. The cells transduced with either siRNA or Trim5 expression constructs were stable high m.o.i lines. After separation by SDS-PAGE and transfer to PVDF, blots were probed with a polyclonal antibody directed against a peptide in the N-terminus of Trim5α.

serves as a control. These were compared to three wild type human cell lines. HT1080, HeLa and TE671, as well as to cells stably expressing HuTrim5α or Owl Monkey Trim5CypA from retroviral vectors (m.o.i. approximately 10). These were probed with an antibody directed against the N-terminus of Trim5α (Figure 7.10).
A strong band was observed at around 55kDa for cells transduced with HuTrim5α and as expected a slightly smaller band for Owl Monkey Trim5CypA. All of the cells showed a faint doublet corresponding to molecular weights been 55 and 60kDa. These however do not seem to correspond to Trim5 as they were (a) apparent in *M.dunni* cells which to not express Trim5 and also in siRNA expressing cells (b) slightly larger than the band corresponding to over-expressed Trim5α. A similar situation applies to a band of approximately 38kDa, present in somewhat greater abundance. No band could be attributed to Trim5 in any wildtype cell line.
Trim5α expression levels in wild type cells therefore appear very low (undetectable) when compared to transduced cells. This experiment was also repeated with 4 other commercially available antibodies with similar results. This observation is not entirely unexpected. Fv1 expression levels are also very low. Low expression is also consistent with the saturability of restriction in these cells.

7. 8 Effect of geldanamycin on restriction by Trim5α

Geldanamycin is well characterised as a potent and specific inhibitor of HSP90. Despite the fact that interaction with other proteins cannot of course be completely ruled out, of more pressing concern is the range of HSP90 client proteins. These interactions range from other chaperone proteins such as HSP70, metabolic enzymes such as Glyceraldehyde 3 phosphate dehydrogenase and structural proteins such as vimentin. Via its actions on HSP90, GA also inhibits cell division and induces apoptosis in a cell type dependent manner. Derivatives of GA are in advanced clinical trials as chemotherapeutic agents. In addition, geldanamycin has also been shown to inhibit the replication of Herpes Simplex Virus, VSV and HIV-1 and although the specific mechanism of inhibition has not been investigated, effects on cell cycle progression have been suggested as important (Li et al., 2004).
Given these broad effects, the results of experiments with GA become very difficult to interpret. However, the use of N and B MLV as restricted virus and non-restricted

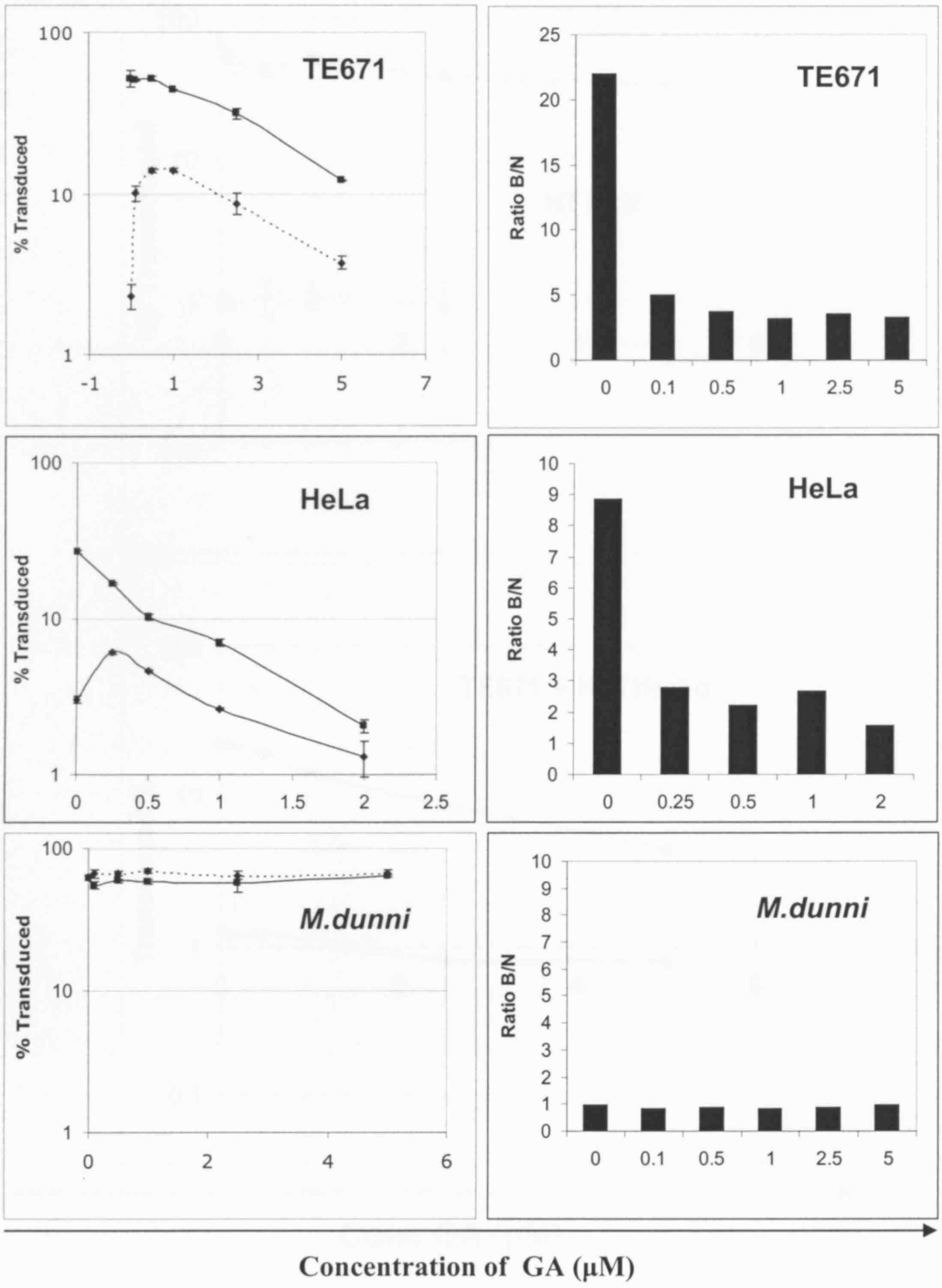

Figure 7.11 Effect of geldanamycin on N-and B-tropic MLV infectivity in TE671, HELA and *M.dunni* cells.
Cells were split at $4x10^4$ cells per well in a 12 well plate 24hrs prior to the experiment. Cells were incubated with geldanamycin (GA) at the indicated concentration for 2 hours before washing twice with PBS followed by replacement of fresh media. Cells were then infected with a fixed dose of either N(♦)- or B(■)- tropic MLV of equal titre. Percentage of successfully transduced cells was determined 2 days later by flow cytometry. Data was plotted either as concentration of GA used/percentage transduced (left column) or GA used/ratio percentage transduced B/N (right column).

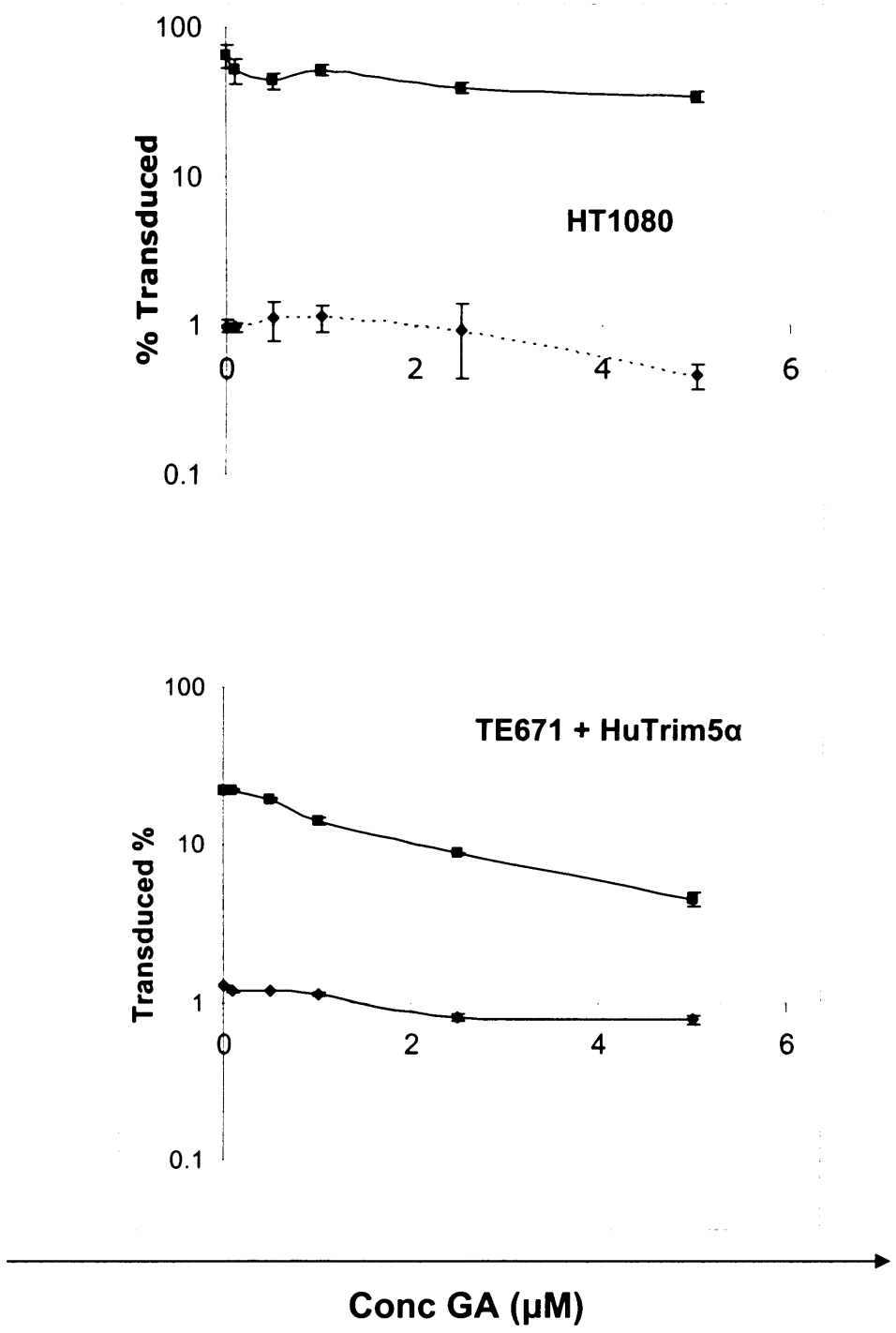

Figure 7.12 Effect of geldanamycin on N-and B-tropic MLV infectivity in TE671 cells over-expressing HuTrim5α and HT1080 cells.
Cells were seeded at $4x10^4$ cells per well in a 12 well plate 24hrs prior to the experiment. Cells were incubated with geldanamycin (GA) at the indicated concentration for 2 hours before washing twice with PBS followed by replacement of fresh media. Cells were then infected with a fixed dose of either N(♦)- or B(■)- tropic MLV of equal titre. Percentage of successfully transduced cells was determined 2 days later by flow cytometry.

control helps to overcome some of these issues, particularly involving effects on cell cycle and virus replication. Any block to cell cycle progression or induced apoptosis will reduce MLV activity as MLV cannot infect non-dividing cells, but any consequence should impact on N and B tropic MLV equally. As described in detail in chapter 2, restriction can be measured as ratio of transduced cells by equal titre N and B MLV. Any drop in restriction is seen by a drop in this ratio. Therefore effects on infectivity not related to restriction can be discounted.

Using this approach, TE671 cells, HeLa cells and *M.dunni cells* were infected with equal amounts of either N or B tropic MLV after 2 hours of pre-treatment with the indicated concentration of GA (Figure 7.11)

As expected, geldanamycin significantly reduced the infectivity of B MLV in both TE671 and HeLa cells. Surprisingly however, at low concentrations GA treatment increased N-MLV titre. This was followed by a drop in titre at higher concentrations. This correlated with significant reduction in cell growth at high concentrations.

As described above, the ratio B/N can be calculated. Untreated TE671 cells had a ratio of 22. With any amount of GA present, this ratio dropped to around 5. N MLV is still restricted relative to B tropic but restriction is much weaker. Untreated HeLa cells began with a ratio of around 9. This dropped to below 3 with 2μM GA.

As a control, non-restricting *M.dunni* cells were also used. There was no change in the ratio B/N. GA also appeared to have little impact on B MLV titre in these cells. There was also very little effect on cell growth.

HT1080 cells restrict N MLV more strongly than either TE671 or HELA cells, with ratio B/N in untreated cells at around 75. Treatment with GA did not significantly relieve restriction (Figure 7.12, top)

To examine the effect on cells over-expressing HuTrim5α, stable TE671 HuTrim5α cells were also tested (Figure 7.12, bottom) In these circumstances, GA had no detectable effect upon restriction, suggesting that the effects on GA can be overcome by over-expression of Trim5α.

7.9 Discussion

The role of Trim5α cytoplasmic bodies is not at all clear. Whether or not they exist when Trim5α is expressed at an endogenous levels is still unknown. Given the very

low endogenous expression levels, a cellular distribution like that observed when over-expressed is perhaps not likely. An absence of highly specific antibodies continues to make answering this question very difficult.

The experiments described here have shown that GFP-Trim5α cytoplasmic bodies are highly motile. Periods of fast, directional movement are interspersed with short saltatory movements and stationary periods. Treatment of cells with nocodazole eliminate the fast, directional movements suggesting that these movements are mediated by the microtubule network. In support of this hypothesis, GFP-Trim5α cytoplasmic bodies appear associated with CFP-α-tubulin when examined by microscopy. Despite this, the fact that nocodazole does not relieve restriction suggests that microtubule based movement in itself is not absolutely required for restriction activity.

The data presented here regarding the effects of GA on this system are complex and difficult to interpret for the reasons already given. Treatment clearly has a dramatic effect upon the localisation over-expressed of Trim5α. GFP signal from cytoplasmic bodies is reduced and peri-nuclear accumulation occurs. This correlates with a rapid reduction in protein levels.

Treatment of TE671 and HeLa cells with GA can relieve restriction. There is little effect on HT1080 cells and no effect upon TE671 cells over-expressing Trim5α. These data could be explained by a reduction in protein levels following treatment with GA, partially overcoming restriction in TE671 and HeLa cells. Restriction in HT1080 cells is stronger and is not overcome. A similar situation applies to TE671 cells over expressing Trim5α.

Although one cannot assume that this phenotype occurs due to a direct interaction between HSP90 and Trim5α (other HSP90 clients could be involved), such an interpretation can explain these data. HSP90 is involved in the folding and maintenance of stability of a number of proteins. Inhibition of HSP90 has been shown to lead to their degradation. This could explain the partial loss of restriction in some wild type cell lines, but not when Trim5α is over-expressed or in cells which more strongly restrict N-MLV. The appearance of a peri-nuclear accumulation is consistent with a mass of unfolded protein awaiting degradation (Kopito, 2000). Interestingly, HSP90 has also been implicated in the microtubule based transport of several proteins (Pratt et al., 2004).

Two groups have argued that cytoplasmic bodies are irrelevant for restriction. One group noted that greater expression levels result in more, larger, cytoplasmic bodies. Lower expression levels apparently did not produce such bodies yet restriction was still observed. The data shown in figure 7.7 show that determination of the existence or absence of cytoplasmic bodies by microscopy is highly subjective. Basic image enhancement is capable of producing entirely different conclusion.

The other group used GA to suggest that the apparent absence of these bodies has no effect on restriction. The data presented here show that this may be a consequence of over expression. Such treatment at wild type expression levels can have consequences for restriction.

It is difficult to clearly establish a role or indeed absence of a role for cytoplasmic bodies in restriction. It may be that the localisation pattern observed in figure 7.1 is partly a consequence of over-expression. An intrinsic ability to self associate may lead to the formation of cytoplasmic bodies when the protein is over-expressed. The bodies may then be transported upon the microtubule network for eventual degradation. Such an interpretation however does not exclude the possibility that the behaviour of the bodies and the fact that they can form is relevant to the mechanism of action of Trim5α. Potential implications of this are discussed in detail in the next chapter.

CHAPTER 8

Discussion

8.1 Binding of Trim5α and Fv1 to Polymeric CA

8.1.1 A mature polymeric CA core is required for incoming virus to interact with either Fv1 or Trim5α

Despite tremendous effort by a number of labs, it has so far proven impossible to show direct binding between Fv1 and MLV CA with any standard technique using monomeric CA. Neither is there any published data showing binding of Trim5α to either HIV-1 or MLV CA using the monomeric protein. The data presented here help to rationalise this absence. CA processing is necessary for assembly into a mature polymeric core. Mutations which block either N or C terminal CA processing inhibit the ability of an incoming virus to interact with either Fv1 or Trim5α in abrogation experiments as do mutations that prevent proper core assembly. In addition, expression of properly processed CA in restricting cells has no effect upon the incoming virus despite being freely distributed throughout the cytoplasm and thus potentially available to interact with restriction factors. Taken together, these data suggest that CA must be in the polymeric form found in a retroviral core in order to interact with either Fv1 or Trim5α.

One might advance three explanations for this requirement which are not necessarily mutually exclusive:

i. The binding site for Fv1 or Trim5α may be generated by the juxtaposition of CA monomers found in the hexameric lattice. Figure 8.1 shows the location of residues on the MLV NTD hexamer known to affect MLV tropism based upon the work of Stevens and co-workers. (Stevens et al., 2004). Residues affecting HIV-1 tropism are also shown on an NTD monomer (Hatziioannou et al., 2004a). These residues are located on upper and outer surfaces of the monomers, potentially available to produce a binding site between adjacent hexamers in the mature lattice or perhaps between monomers when juxtaposed within a hexamer. Arguing against an inter-

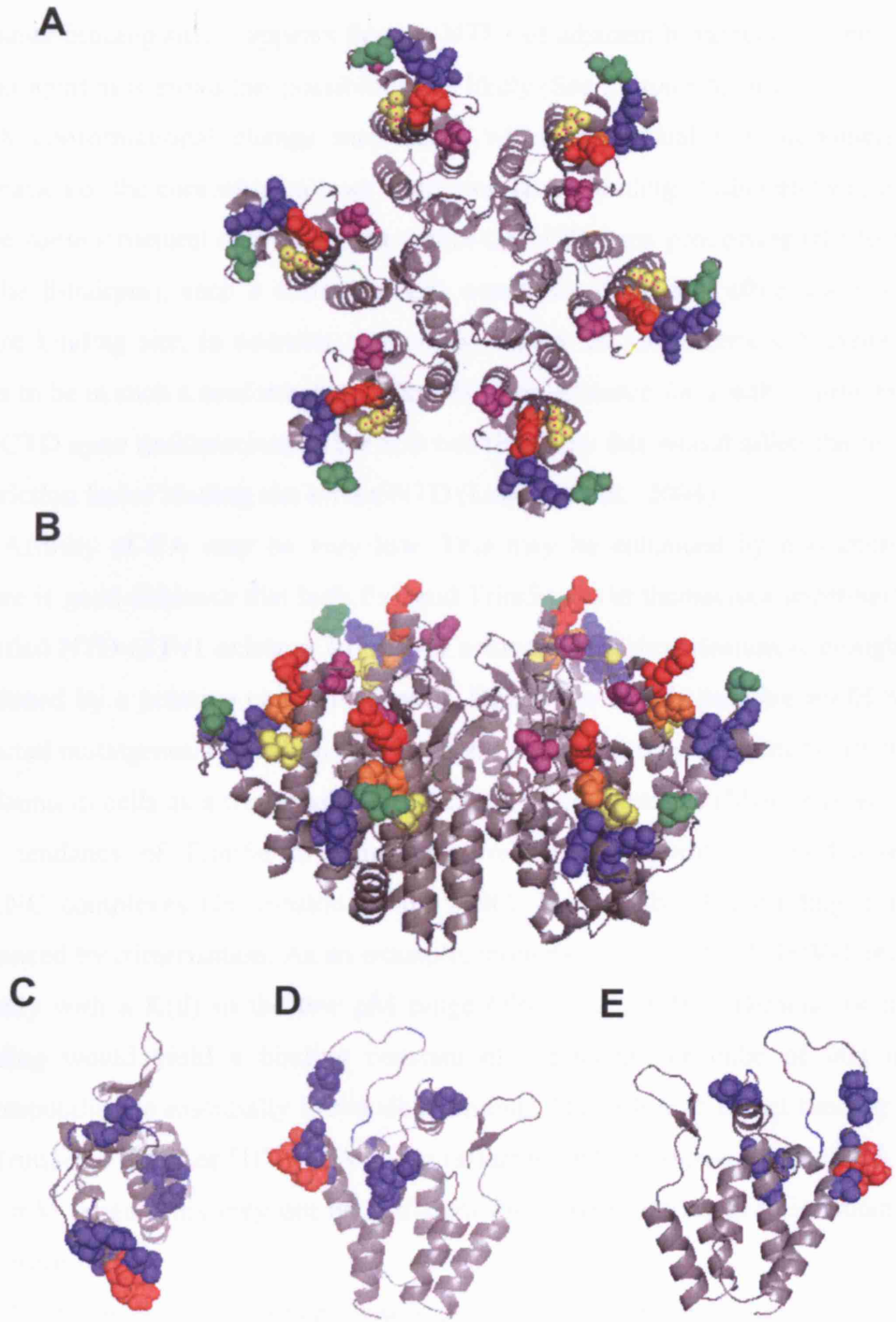

Figure 8.1 Species specific tropism determinant on MLV and HIV CA
(a) Residues described by Stevens {Stevens, 2004 #24} to affect tropism are shown on the MLV NTD hexamer are. Position 110 is coloured red, 82 is green, 92-95 are blue, 105 is purple, 114 is orange, and 117 is yellow. (b) The hexamer is tilted to better show the location of key residues. (c-e) Residues shown to affect HIV tropism by Hatziioannou {Hatziioannou, 2004 #282} are shown coloured on a HIV CA monomer. For reference, E79 and R82 (which also affected tropism) are shown in red. (c) 'top' (e) 'right' side (f) 'left' side

hexamer binding site, it appears that the NTDs of adjacent hexamers may be situated so far apart as to make this possibility less likely (See *Chapter 6, Discussion*).

ii. A conformational change may occur within individual CA monomers upon formation of the core which allows restriction factor binding. Although there is likely to be some structural rearrangement within the NTD upon processing (the formation of the β-hairpin), such a rearrangement would not obviously affect the restriction factor binding site. In addition, one would expect the monomeric CA expressed in cells to be in such a conformation. There is some evidence for a shift in orientation of the CTD upon multimerisation but it is not clear how this would affect the presumed restriction factor binding site on the NTD (Lanman et al., 2004).

iii. Affinity of CA may be very low. This may be enhanced by multimerisation. There is good evidence that both Fv1 and Trim5α are in themselves multimeric. The purified NTD of Fv1 exists as a dimer in solution. This dimerisation is thought to be mediated by a putative coiled coil motif. Disruption of this putative mofif by site-directed mutatgenesis can abolish restriction (Yap and Stoye, submitted). Trim5α can be found in cells as a trimer after crosslinking of cell extracts (Mische et al., 2005). The tendancy of Trim5α to trimerise correlates with ability to bind assembled CA:NC complexes (Javanbakht et al., 2006). It could be that binding avidity is enhanced by trimerisation. As an example, monomeric CypA binds HIV-1 relatively weakly with a K(d) in the low μM range (Yoo et al., 1997). Dimeric or trimeric binding would yield a binding constant of the square or cube of this number corresponding to essentially irreversible binding. The failure to detect binding of Fv1 or Trim5α to MLV or HIV-1 CA might reflect a binding constant in the high μM or low mM range. This may not be sufficient for activity until avidity is enhanced by trimerisation.

It is therefore likely that both polymerisation of CA and trimerisation/dimerisation of Trim5α/Fv1 are both required for successful restriction. Work by Aiken and colleagues undertook a similar study to the one described here, using HIV-1 and Trim5CypA. They also concluded that a properly formed CA core was required for abrogation of restriction. The binding site for Trim5CypA is monomeric – the CypA binding loop. Despite this, a core of proper stability was still required for abrogation (Forshey et al., 2005) suggesting that polymeric CA is essential. Resolving the question of the precise Trim5α binding site will most likely require structural data showing Trim5α in complex with polymeric CA. Despite this, it seems clear that

multimerisation of restriction factors, which would enhance their avidity for CA, is essential for their activity.

One can speculate that there may be good evolutionary reasons why Trim5α and Fv1 may bind the polymeric form of CA rather than the monomeric. It is clear that both Trim5α and Fv1 are both expressed at extremely low levels. (Yap and Stoye, 2003). Both proteins are prone to aggregate (discussed later). In these circumstances, high expression may be detrimental to the host cell. A protein expressed at very low levels may by necessity have to be extremely selective in the CA it targets. It is clear that at least 1/2 of CA within a virion is not found in a polymeric core (Briggs et al., 2004; Li et al., 2000). In addition, at least some of the CA from the core may be lost relatively quickly (Fassati and Goff, 2001). If monomeric CA were bound with high affinity, then the restriction factor could be more easily saturated. Under these circumstances it may be necessary to selectively and efficiently target the assembled CA found on a core.

8.1.2 Experiments showing binding of Trim5α to polymeric CA

Chapter 5 described an experiment designed to detect binding of Trim5α to HIV CA core material derived from detergent stripped virions. Under these circumstance, binding was not detected. However, during the course of these studies, two other groups have successfully shown association between MLV/HIV-1 CA and Trim5α. Work from the Luban group showed that addition of MLV virions to a cell extract containing GST-tagged Trim5α and mild detergent allowed association between CA and human GST-Trim5α. GST-Trim5α complexes could be collected on glutathione coated beads. Importantly, this occurred for N-MLV but not B-MLV. However, the authors were unable to detect an interaction between HIV-1 and Trim5α under the same conditions (Sebastian and Luban, 2005).

In a separate study, the Sodroski group successfully showed binding of Trim5α to *in vitro* assembled CA:NC complexes (Stremlau et al., 2006). In high salt conditions and supplemented with nucleic acid, bacterially expressed and purified HIV CA-NC protein polymerises to form hexameric assemblies, the majority in the form of tubes but also in the form of conical cores (Ganser et al., 1999). Using this material, the authors were able to bind restricting Trim5α in cell extracts, centrifuge assembled

tubes/cores through a sucrose cushion and show Trim5α in association with pelleted tubes. As predicted, this occurred in a species specific manner.

These data support that presented in Chapters 3 and 4 suggesting that Trim5α can bind polymeric CA assemblies. It is not however clear why the binding experiment described in Chapter 5 was unsuccessful. Experimental conditions were very similar although not identical. The most obvious differences lie in the preparation of the CA material and also the NC domain fused to CA. The authors have not reported experiments using polymerised CA alone. It is possible that the presence of NC in some way modifies the CA assembly allowing it to bind Trim5α. However, this does seem unlikely as the authentic target for Trim5α is an incoming HIV core presumably consisting of fully processed CA. We are seeking to reproduce the work of Stremlau *et al.* with purified CA:NC and extend it to include CA alone.

One possible difference between the CA:NC material and the virion core material is contact with CypA and the conformation of the G89-P90 bond. It is possible that the conformation of this bond in the bacterially produced *in vitro* assemblies, which has no contact with CypA prior to incubation in cell extracts, differs from that within a HIV core. During a normal infection both producer and target cell CypA bind CA. The HIV-1 virions here certainly incorporate CypA and therefore have had contacts with one source of CypA lacking in the bacterially produced material.

Target cell CypA in particular is important for infectivity and can affect restriction (Franke et al., 1994; Hatziioannou et al., 2005; Sokolskaja et al., 2004; Towers et al., 2003). It may not be active in the cell extracts (which correspond to target cells), used in these experiments. Such a hypothesis could explain also why the Luban group were unable to show binding of Trim5α to HIV CA in their assay when it was possible with the non-CypA binding MLV.

Despite these plausible explanations, the precise effects of CypA on the material used in these experiments is not at all clear. It is therefore difficult to develop a clear model as to how it actions might affect binding Trim5α. To circumvent this issue, it may be useful to repeat these experiments with a lentiviral CA that does not bind CypA, either using the HIV-1 G89V mutant or perhaps EIAV detergent stripped cores which do not bind CypA but are restricted by several isoforms of Trim5α.

8.2 Towards a mechanism of action for capsid dependent restriction factors

8.2.1 Artificial CypA fusion proteins can act as restriction factors

Before discussing new data relating to the mechanism of action of restriction factors, it is first important to describe some recent work by our laboratory examining the antiretroviral activity of novel Trim-CypA fusion proteins. After the discovery that Trim5CypA could restrict HIV-1 and that CypA appeared to function as the CA binding domain in place of the SPRY domain, experiments were undertaken to determine whether other Trim proteins could also restrict HIV-1 when fused to CypA. Fusions were made between the RBCC domains of Trim1, Trim18, Trim19 with CypA. All were found to restrict HIV-1 in a CypA binding dependent manner – the CypA binding mutant G89V was unaffected by any fusion protein and the presence of the competitive inhibitor cyclosporine relieved restriction. (Yap et al., 2006). Later work by Bieniasz and colleagues showed that this was also the case for Trims 6, 21, 22 and 34 (Perez-Caballero et al., 2005a).

For Trim1 and Trim18, two fusion proteins were made, one fused directly after the predicted coiled-coil region, the other including an extra 195/165 amino acids of Trim sequence. Curiously, the longer proteins (T1CL, T18CL) were found to block HIV-1 prior to reverse transcription (analogous to Trim5α and Trim5CypA) whereas the shorter proteins (T1CS, T18CS) blocked at a stage after reverse transcription, as did the Trim19 (PML) fusion. The production of 2LTR circles was also unaffected by these proteins suggesting that the virus could still enter the nucleus (Brown, 1997). Confusingly, if either of the short forms were tagged on either their N or C termini with HA or GFP, the timing of block reverted to the early stage prior to reverse transcription.

Despite the altered timing of block, all were shown to bind the virus soon after entry. Cyclosporine A was only capable of relieving restriction when added within 1 hour of cell entry suggesting that the early binding of the Trim-CypA fusion protein could produce two different phenotypic consequences - One where the virus progresses through reverse transcription but is incapable of proper integration and another where it is blocked prior to reverse transcription.

Expanding this study further, it was shown that if the NTD of Fv1 is fused to CypA it is capable of restricting HIV-1 in a manner dependent upon its ability to multimerise (disruption of the putative coiled-coil abolished restriction). Fv1CypA did reduce levels of early reverse transcription products although not to the same degree is Trim5α. Finally, fusion of CypA to Arfaptin 2 – a protein entirely unrelated to either Trim proteins or Fv1 could also result in restriction of HIV-1 – again dependent upon binding within the first hour of infection but manifesting itself at a very late stage after reverse transcription (Yap and Stoye, submitted). Arfaptin shares with both Trim proteins and Fv1 the ability to multimerise – in this case as a dimer (Tarricone et al., 2001).

8.2.2 Multimerisation and CA binding

Taken together, these data suggest at least two minimum requirements for restriction of HIV-1. The first is an ability to bind the virus CA protein. The second is the ability to multimerise. Multimerisation probably contributes to higher avidity binding as discussed earlier. It is not clear whether it contributes to the antiviral activity of restriction factors in any other way. Regardless, disruption of this ability abolishes restriction in every case where it has been attempted (Mische et al., 2005)(Yap and Stoye, submitted). In the case of Trim5α, the dominant negative effect of Trim5γ which possess a coiled-coil domain but lacks a binding domain underlies the importance of multimerisation.

In order to bind two or more sites simultaneously on the surface of a viral core, the CA binding domains would have to be appropriately spaced and positioned. In the case of CypA-arfaptin, the arfaptin dimer is an anti-parallel 'arch shaped' extended helical structure. Attachment of CypA domains to the N-termini of the dimer is predicted to position them at either end of this arch (Tarricone et al., 2001). Recent data suggests that the Fv1 NTD dimer also forms an extended helical structure although the orientation of the monomer is not clear (Bishop et al., 2006b). The association of Trim5α in a trimer via the coiled-coil domain could also position the SPRY domains in the same orientation, potentially available to bind a 3 fold axis of symmetry within the hexamer lattice (Mische et al., 2005).

Importantly, the data from the Arfaptin constructs suggest that no effector activity is *absolutely required* for CA based restriction. This however does not exclude the

possibility that some effector/enzymatic activity contributes or is required for the full activity of Trim5α, Fv1 or Trim5CypA.

8.2.3 Uncoating, degradation or sequestration?

During the course of these studies, two key papers have been published suggesting potential mechanisms of action for Trim5α and Trim5CypA. One was from the Hope group (Wu et al., 2006). Prior to the publication of this paper, a number of groups had suggested that presence of the RING-domain with its associated ubiquitin ligase activity, implying a role for the proteasome, plays no part in the activity of Trim5 (Perez-Caballero et al., 2005b; Stremlau et al., 2006). This appears not to be the case. Data from the Hope group showed that when proteasome inhibitors are added to HIV-1 restricting cells, whilst overall restriction activity is unaffected, the block to infection now occurs after reverse transcription, suggesting that proteasomal degradation in some way mediates an early block by Trim5α, but in its absence, a later block still remains. This is reminiscent of the CypA-Arfaptin fusion protein where no clear effector activity exists yet the virus is still blocked at a late stage. Interestingly, when pre-integration complexes are isolated from cells treated with proteasome inhibitors, they are still competent for *in vitro* integration reactions in a manner analogous to MLV restriction by Fv1. This may hint at a role for the stable sequestration of the viral core in these circumstances.

In parallel to these studies, work aimed at determining the fate of CA in restricting cells by the Sodroski group suggested that rather than proteasomal degradation, the mechanism of action of Trim5α was the accelerated uncoating of the incoming viral core. This accelerated uncoating was suggested to prevent the process of reverse transcription thereby blocking infection of the incoming virus (Stremlau et al., 2006). The authors showed that when infected overexpressing Trim5α cells are lysed and the RTCs/PICs are isolated by centrifugation through a sucrose cushion, less CA is found in association with the pelletable complex. In the case of MLV (but not HIV-1) it was instead found free in the supernatant. An attempt was made here to reproduce this data (Chapter 5, Figure 5.8). Indeed, less pelletable CA material was observed however this did not correspond to an increase in free CA for either HIV or MLV in these assays. In addition, a similar effect occurred for MLV in cells expressing Fv1 despite the fact that MLV blocks the virus after reverse transcription.

In all circumstances, total CA as determined by western blot appeared to remain unchanged. However, given the very small proportion of CA (<5%) that is pelletable compared to the total CA even in non-resticted cells, any small decrease in total CA due to degradation of the pelletable core material would almost certainly be undetectable.

The evidence for an accelerated uncoating leading to the block to reverse transcription is therefore not absolute. Other mechanisms may also play a role. Taking the data from both papers and Chapter 5 together, it appears very possible that the primcipal mechanism of action of both Fv1 and Trim5α involves the degradation of the incoming core. Despite this, in the case of Trim5α,neither ubiqutin ligase or cellular proteasomal activity is essential for restriction. There no indication that Fv1 has any direct link to ubiqutin mediated proteasomal degradation. This may suggest that the binding of both Fv1 and Trim5α to CA is sufficient to prevent infection either sequestering the virus and/or leading to its degradation by a non-proteasomal mechanism. Such a model could explain why in the case of both Fv1 or Trim5α with proteasome inhibitors, the virus is able to complete the process of reverse transcription.

8.2.4 A role for HSP90 as a Trim5α chaperone

The data described in Chapter 7 hint at a role for HSP90 in the functional activity of Trim5α. HSP90 has a many roles in the cell imparted by its range of its client proteins. Its specific role in the case of each client protein can vary. For many, HSP90 functions as a classic chaperone, assisting the proper folding of nascent proteins in partnership with HSP70 (Wegele et al., 2004). It is also involved in microtubule based transport of steroid receptors (Pratt et al., 2004). For others is appears to be necessary for the maintenance of stability of its client proteins (Marcu et al., 2002; Zhang et al., 2006b). HSP90, like other chaperones is also found in association with degradative structures such as aggresomes (Garcia-Mata et al., 2002).

Inhibition of HSP90 activity by geldanamycin results in lower expression levels of Trim5α and peri-nuclear accumulation of fluorescently tagged material in stable cell lines. At endogenous expression levels of Trim5α, GA can relieve MLV restriction.

The mechanism by which this might occur is not clear. Trim5α is a complex multidomain protein and so may require a chaperone for correct folding. Data suggests that Trim5α is turned over rapidly (Diaz-Griffero et al., 2006) therefore inhibition of its proper synthesis may result in a rapid reduction of expression levels. Alternatively, HSP90 may be required for proper transport of Trim5α. Finally, HSP90 may be required for the degradation of Trim5α aggregates. As described below, this could also have implications for its activity. Despite all of these plausible connections, one must be cautious is ascribing any direct link between HSP90 and Trim5α based upon drug studies alone. It may be that another Trim5α client protein interacts with Trim5α or that geldanamycin can have other, as yet undefined, effects on the cell.

8.2.5 Proteosomal and aggresomal mediated degradation and the nature of Trim5α cytoplasmic bodies

Recent work has suggested that Trim5α is rapidly produced and degraded within the cell. Treatment of cells with the translation inhibitor cyclohexamide causes a rapid decrease in expression levels of even over-expressed Trim5α. The half life of Trim5α within the cell has been estimated at 50-60 minutes (T. Hope, personal communication (Diaz-Griffero et al., 2006). In the presence of proteasomal inhibitors, Trim5α is polyubiquitinated (Diaz-Griffero et al., 2006). The short half-life and polyubiquitination is dependent on the presence of the RING domain (which possesses E3 ubiquitin ligase activity), suggesting that Trim5α may self-ubiquitinate triggering its own degradation via the proteasome. There is no evidence that Trim5α is capable of ubiquitinating CA.

When proteins targeted for degradation are not degraded rapidly enough by the proteasome, or the capacity of proteasome is exceeded they form aggregates. This is especially true if proteins are partially unfolded (Wickner et al., 1999). These aggregates are transported in a microtubule dependent manner to a degradative structure found near the microtubule organisation centre (MTOC) known as the aggresome (Garcia-Mata et al., 1999),(Garcia-Mata et al., 1999). The cytoplasmic bodies formed by Trim5α in the stable cell lines described in chapter 7 strongly resemble the aggresomal precursors. First, by their microtubule based movement shown here, and second because when Trim5α is very highly over expressed by

transfection followed treatment with sodium butryate, large perinuclear accumulations of protein are observed which bear the hallmarks of aggresomes such as co-localisation with HSP70 and caging by vimentin fibres (Diaz-Griffero et al., 2006; Perez-Caballero et al., 2005b; Song et al., 2005a)(T.Hope, personal communication). Dramatic over expression of Trim5α may overcome the ability of the cell to degrade Trim5α in a controlled fashion and instead proteins accumulate in aggresomes.

These accumulations are not static structures and the cell appears to have a number of ways of dealing with them. Proteasomes are recruited to the area to assist in the degradation of the aggregated proteins, particularly those which are ubiquitinated Perhaps more important is a second pathway known as autophagy. In this pathway, the aggresomal proteins are engulfed into a membrane bound organelle known as an autophagosome which later fuses with lysosomes leading to proteolytic degradation of the contents (Lee et al., 2002).

Recently, a consensus has begun to emerge that Trim5α cytoplasmic bodies are almost certainly aggresomes or aggresomal precursors destined for degradation (Diaz-Griffero et al., 2006; Perez-Caballero et al., 2005b; Song et al., 2005a). The data here described in chapter 7 showing their movement on microtubules is consistent with this hypothesis. Given the very low endogenous expression, one might suggest that the formation of these bodies is irrelevant to the mechanism of action of Trim5α. However, an alternative hypothesis does present itself.

8.2.6 A model of Trim5α restriction of HIV-1 and MLV

When a virus core enters the cytoplasm of the host cell, it must strongly resemble a large protein aggregate. It appears however that in general it is not treated as such. The virus is not usually destroyed by the host degradative machinery, although treatment of non-restricting cells with the proteasomal inhibitor MG132 can enhance HIV infectivity in non-restricting cells, perhaps suggesting that at least a small proportion of incoming viruses are dealt with in this manner (Schwartz et al., 1998).

The binding of Trim5α which is itself targeted for degradation, to the capsid protein, may increase the visibility of the virus to the host cell degradative machinery thus leading to a block in infection. This might of course involve the proteasome however the activity of the proteasome would not be essential. In a proteasome inhibited cell,

the viral core would eventually be degraded by autophagy. This may involve a period of sequestration where the virus is unable to continue to a productive infection but is still capable of continuing the process of reverse transcription. Such a model is illustrated in figure 8.2.

In the model shown in figure 8.2, the whole virus core is shown in association with the proteasome. It is difficult to imagine exactly how a proteasome might deal with an intact virus. A virus is certainly larger and proteasomes are known to deal with only single polypeptide chains. Degradation of CA may therefore have to involve some process that would appear as uncoating to produce monomeric CA.

This hypothesis has the advantage that it can explain the activity of both Fv1 and the artificial CypA fusion proteins. Fv1 and Arfaptin have no clear enzymatic activity that would target them for degradation. This may however not be essential. Simply binding multiple copies of a protein to the surface of an incoming virion may make it appear more likely to be treated as a protein aggregate, leading to its sequestration and eventual degradation. Consistent with this hypothesis, most of these proteins lacking ubiquitin ligase activity blocked the virus at a stage after reverse transcription. The fusions that were capable of an early block (T1CL and T18CL) do possess ring domains (although the shorter forms which block late also do) and ubiquitin ligase activity perhaps targeting them for more efficient degradation. Although originally selected as a protein with no clear link to the activities of Fv1 or Trim5α, except in its capacity to self-assocaiate, the CypA-arfaptin 2 may also owe its activity to aggregation. Arfaptin 2 has been linked with protein aggregation. Arfaptin 2 associates with aggregates of huntingtin protein and induces their formation in cultured cells (Peters et al., 2002).

8.2.7 Sequestration and aggregation as a general antiviral response

That concept the self-association of a Trim protein can be functional is not unprecedented. Trim19 (PML) is the primcipal component of bodies within the nucleus known PML or ND10 bodies. A number of viral genomes have been observed to associate with these bodies including herpes simplex virus type 1 (HSV-1), SV40, polyomavirus and parvovirus AAV adeno-assocated virus (Everett, 2006; Everett and Murray, 2005; Fraefel et al., 2004). PML is up-regulated in response to

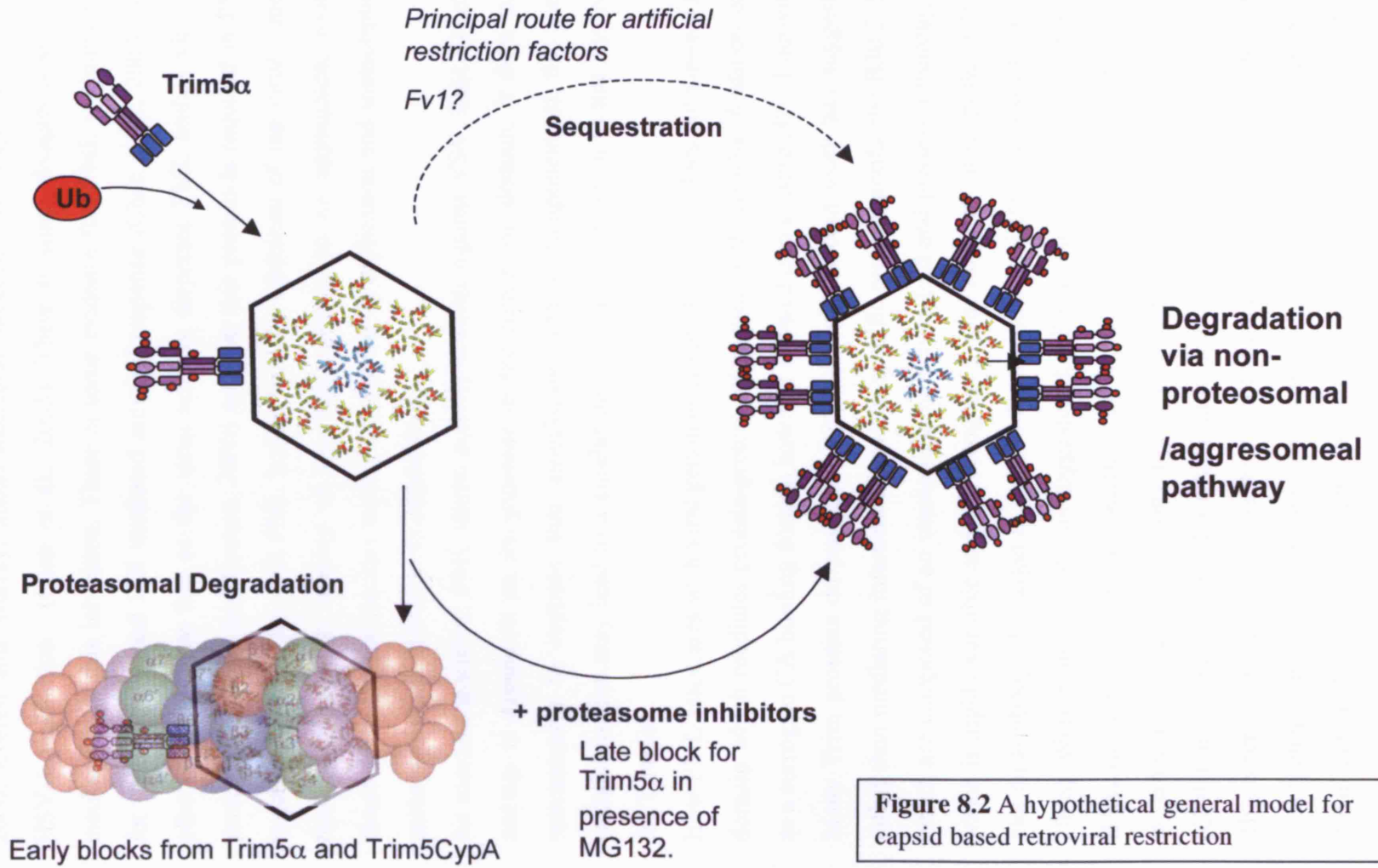

Figure 8.2 A hypothetical general model for capsid based retroviral restriction

interferon. HSV-1 infection can induce the formation new of ND10 bodies (Everett, 2006; Everett and Murray, 2005) Interferon induced PML expression can inhibit HSV-1 replication (Chee et al., 2003). There is some disagreement as to the mechanism of this inhibition. There is some evidence that PML is simply required for inducing a broad IFN mediated antiviral response (Chee et al., 2003), whereas others would argue that as the virus actively disperses PML bodies via its ICP0 protein (Parkinson and Everett, 2000) and that this protein is required in PML up-regulated cells. virus, that PML bodies mediate inhibition of the virus. One might then speculate that binding of the viral genome to an aggregated, multimeric structure within the nucleus would then inhibit its replication and transcription, and perhaps eventually lead to its degradation.

As mentioned earlier, PML shows activity towards against VSV. Like Trim5α, this activity is dependent on the presence of the coiled coil domain. It does not seem unreasonable to suppose that association with a component of the virus and multimerisation may lead to a similar mechanism of inhibition to that hypothesised for Trim5α.

How PML associates with viral genomes is not clear. PML does not possess a B30.2 domain which mediates protein-protein interaction with Trim5α. Addition of CypA as a surrogate CA binding domain however allowed it to acquire HIV-1 activity.

Many Trim proteins do possess a B30.2 domain. Recent work has suggested that rather than mediating interaction between specific protein motifs, the B30.2 domains which are composed of an immunoglobulin type fold and possess a binding surface which is highly variable and can recognise a wide range of target proteins (although like an antibody, the target is specific for an individual B30.2 domain) (Woo et al., 2006). Work from this lab has suggested that 3 variable loops on one surface of the B30.2 domain can alter the specificity of Trim5α restriction (Ohkura et al., 2006). It is tempting to draw an analogy to the complementarity determining region loops (CDR) of an immunoglobulin which allow interaction with a huge range of targets. This offers the potential that via their ability to multimerise and then further associate into higher order structures, the Trim family of proteins could be responsible for a broad antiviral activity, with specificity depending upon the binding domain they possess.

8.3 Future work

8.3.1 Restriction factor/CA binding

Both published data from other groups and the data presented here show some inconsistencies. It will be important to understand why HIV cores isolated from detergent stripped virions cannot be shown to bind Trim5α. This may reveal important information about the nature of the interaction. An essential first step in this process will be a direct comparison of the binding abilities between the different forms of CA in identical quantity and identical experimental conditions. If HIV cores still prove unable to bind Trim5α in those circumstances then clearly the CA found within them in some way differs from that found in bacterially expressed, *in vitro* polymerised tubes. Determining whether this difference results from altered lattice arrangement, stability or modification by CypA will be critical to understanding restriction factor binding. Expansion of these studies to non-CypA binding viruses where cores can be isolated such as EIAV will be helpful in this process. Where possible, it will also be essential to expand binding experiments to include Fv1 and MLV.

8.3.2 Modification of human Trim5α specificity

If any of the mechanisms of action proposed for restriction factors here are correct, then the prospect of any small molecule therapeutic intervention against HIV-1 that is based upon them is slim. One possibility does however exist. Given that all that is required for human Trim5α to bind and restrict HIV-1 is a single amino acid change, it may be possible to modify the binding of endogenous human Trim5α using a small molecule allowing it to bind HIV-1 CA. One possibility would be to obtain good structural data showing the Trim5α binding site leading to structure based drug design. However in the absence of such structural data, high throughput screens may be an alternative. Binding of Trim5α to CA-NC and virions has been shown. If these assays could be transferred to a plate based ELISA system, potentially a huge number of molecules could be screened for activity.

8.3.3 The mechanism of action of Trim5α

The model described in the previous section attempting to explain the action of CA based restriction factors, whilst compelling, is far from established. Testing such a model will be difficult. It will be necessary to perturb the degradative pathways of cells in more specific ways that simply the use of proteasome inhibitors. It may be important to selectively target aspects of the proteasomal and lysosome mediated degradative machinery and follow the fate of the viral CA and genome in restricting cells using assays such as that described in Figure 5.8.

The model described here predicts that if a restriction factor is itself targeted for degradation, then the virus may be blocked earlier in its lifecycle. It maybe that if the RBCC of Trim5α was fused to Fv1, then Fv1 could perhaps block MLV earlier.

8.3.4 Capsid as a target for drug development

The data presented in Chapter 6 show that mutation of either of two residues on the outer surface of a HIV-1 virion by altering the charge on those residues can essentially abolish the infectivity of HIV-1. Although the exact cause of this deficiency is not clear, these mutations most likely affect the stability or formation of a mature CA core, perhaps interfering with an NTD-CTD interaction. That fact that a relatively small disruption to an exposed region of CA can have such a profound impact upon the virus makes this an exciting prospect as a drug target. One might imagine that this region would be particularly accessible to a small molecule antagonist of core formation/stability both in the cell and the virion.

Most studies examining MLV and HIV-1 assembly have used alanine mutagenesis to identify the regions of the proteins involved. For many proteins this may be sufficient however for a multimeric assembly with numerous and possibly redundant contacts, the data shown here suggest that mutations that alter charges on CA residues can uncover interesting new phenotypes and new interaction domains.

Finally, assays to measure the assembly and disassembly rates of *in vitro* polymerised CA would help in a screen of molecules which may affect many aspects of this process although perhaps more importantly, a clearer description of the inter molecular contacts which make up the CA core would aid in drug design.

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APPENDIX 1 - PUBLICATIONS

The work described here has contributed to a number of publications. Two are in print and are included in the following pages. Pagination shown is that of the journal.

Dodding, MP., Bock, M., Yap, MW., Stoye J.P. (2005) Capsid processing requirements for abrogation of Fv1 and Ref1 restriction. *J.Virol*, **80,** 4061-4067

Yap, MW., Dodding, M.P., Stoye, J.P. (2006) Trim-cyclophilin A fusion proteins can restrict human immunodeficiency virus type 1 infection at two distinct phases in the viral life cycle. *J.Virol*, **80,** 10571-7